Investigations on the epidemiology of blood parasites of small mammals with special reference to piroplasms.

Young, A S

7. Laboratory experiments and maintenance of I. trianguliceps.

7.1. Factors affecting the development of I. trianguliceps.

7.11. Oviposition.

Engorged adult ♀♀ were collected off small mammals as described in general methods and in a few cases were fed experimentally on laboratory mice and rats. They were washed in Mycostatin (500 units/ml.) to prevent the growth of fungi, weighed immediately to the nearest mg. and then placed in tubes 70 x 20 mm. thinly coated internally with plaster of paris. These tubes had previously been autoclaved at 15 p.s.i. for 15 minutes and moistened with Mycostatin. The opening of the tubes was covered with 50µm nylon mesh screening silk which allowed the free flow of air so that the humidities were equalised on the inside and outside. These tubes were then placed in a Museum Jar 20 cm . high which had been filled to a depth of 5 cm. with plaster of paris, painted with black paint to stop the penetration of light and autoclaved at 15 p.s.i. for 15 minutes. The Museum Jar was then moistened with the Mycostatin solution. The plaster of paris removed the danger of condensation on the surface of the glass and maintained a 100% R.H. when moist. These containers were kept at 0°C, 10°C, 15°C and 22°C in constant temperature cabinets. Some adult ♀♀ were kept at room temperature but exposed to 16-8 hours light-dark periods instead of continuous darkness.

The adult ♀♀ were inspected every day without unduly disturbing them and the numbers of eggs produced were counted. The adult ♀♀ were very susceptible to fungi and large losses were sustained during early experiments until antiseptic conditions were used.

Light appeared to have a disturbing effect on adult ♀♀ since they were seen to move continuously and appeared to be searching for a site away from the light. In only one case out of four did adult ♀♀ in the light produce eggs and then the egg production was much lower than in similar conditions in the dark. If the adult ♀♀ were replaced in the dark oviposition continued normally.

During the first few days in their tubes the ticks were mobile but just before oviposition the adult ♀♀ remained quite still.

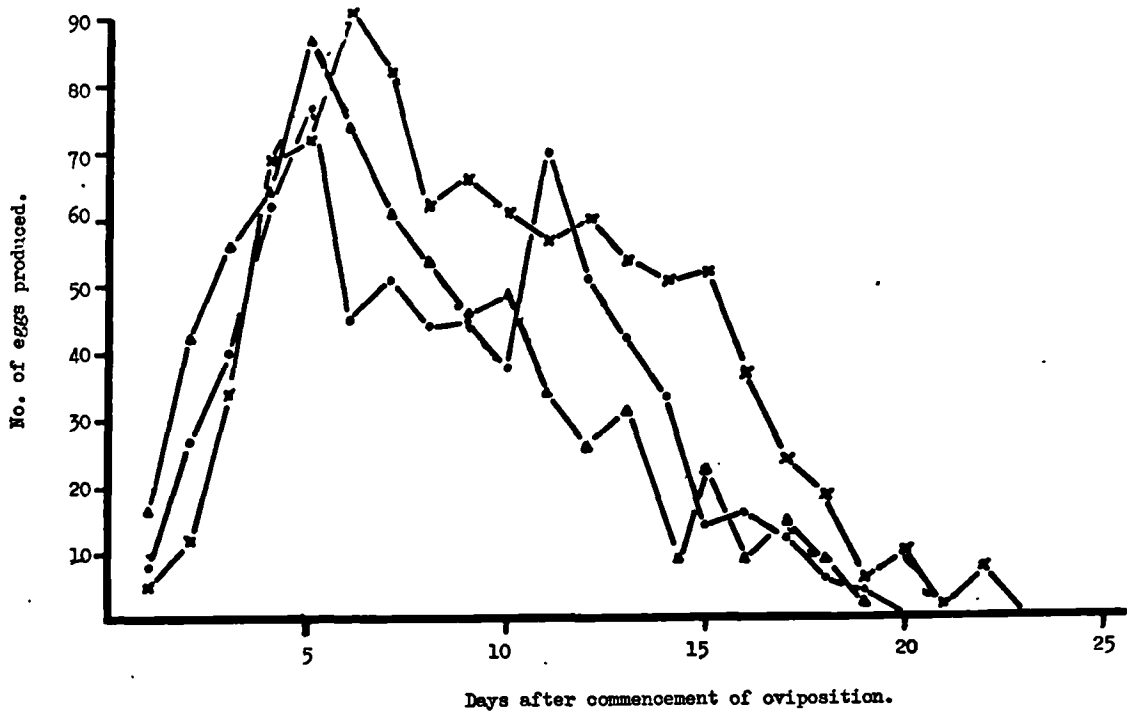
If the adult ♀♀ were left undisturbed they tended to produce eggs in one clump. No oviposition occurred if the adults were kept at 0°C (Table 41). Oviposition occurred normally between 10 and 26°C although in these experiments the limits of development were not defined. The pre-oviposition period varied considerably even at the same temperature. At 22°C, the pre-oviposition period varied between 20 and 114 days. There is some evidence that the period of the year the female fed affected the pre-oviposition period. In November-December (two cases) the adult ♀♀ took a long time to begin oviposition even when the temperature was 22°C, but during the Spring the pre-oviposition period was short (eight cases) while in the late Summer the oviposition period was in between (Table 41a).

The total egg production varied from 60 to 1,006 and was dependent on the weight of engorged females. The adult ticks had to weigh at least 20-35 mg. before they produced eggs. At 22°C, the maximum daily egg production occurred after four to six days when the maximum daily production was 109 eggs per day. Hence it can be calculated that an egg would be laid every twelve minutes

TABLE 41 : OVIPOSITION OF I. TRIANGULICEPS AT A HUMIDITY OF 100%

Temperature °C	Weight mg.	Host	No. Eggs Produced	Preoviposition Period
22	106	Apodemus	1,120	114
22	99	Apodemus	970	91
22	101	Clethrionomys	960	26
22	95	Mouse	Died	23
22	65	Clethrionomys	621	26
22	54	Apodemus	Died	21
22	103	Apodemus	1,030	22
22	34	Clethrionomys	82	29
22	89	Apodemus	862	21
15	93	Mouse	857	41
15	96	Apodemus	968	37
15	116	Clethrionomys	1,021	46
10	69	Apodemus	435	56
10	88	Clethrionomys	587	49
0	102	Apodemus	-	-
0	91	Apodemus	-	-
0	94	Clethrionomys	-	-

Fig. 89: Oviposition of I. trianguliceps: 3 Females at 22°C.



during this period of maximum egg production. The oviposition period varied between 19 and 26 days at 22°C. (Fig. 89).

7.1.2. Hatching.

Hatching was observed to occur above 90% R.H. and below this humidity the eggs became shrivelled and died. At 26°C, 100% R.H., the hatching period was between 28 and 34 days while at 18°C, 100% R.H. the period was between 35 and 48 days.

7.1.3. Moulting.

When engorged ticks were detached from hosts two distinct periods were seen before the completion of moulting. The ticks were active for a few days when they appeared to be searching for a crevice since they tended to come to rest in bubbles in the plaster of paris if these were available. The ticks then remained inactive until moulting was complete. The moulting period was considered the period from detachment from the host to the emergence of the next stage.

Moulting was seen to occur in humidities down to 85% below which the ticks became dehydrated and died. Above this relative humidity ticks moulted normally. No moulting of engorged larvae and nymphs was observed at 0°C (Fig. 90) but when they were later placed in a higher temperature they moulted normally. The temperature limit of moulting appeared to be between 7 and 10°C in larvae, and 10 to 15°C in nymphs. With the increase of temperature the moulting period was greatly reduced to a minimum of 17 days and 22 days for larvae and nymphs respectively at 26°C. However, at particular temperatures considerable variation in the moulting

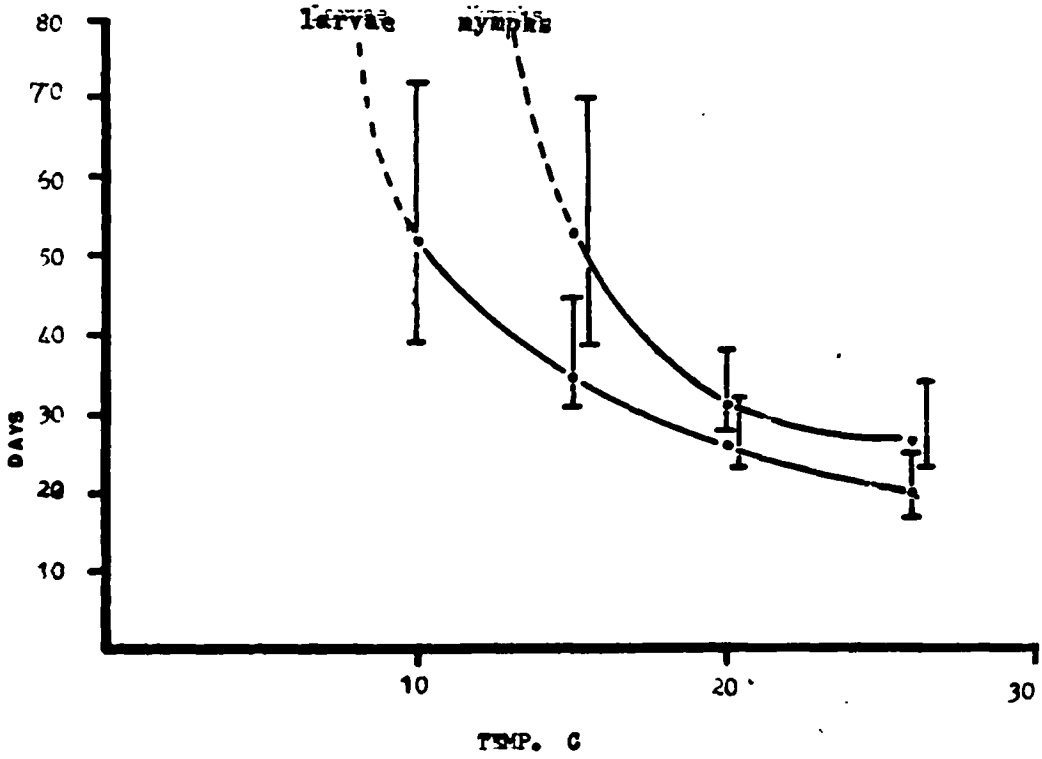


Fig. 90: Period taken for I. trianguliceps to moult at different temperatures.

period occurred.

7.1.4. Survival of unfed stages of I. trianguliceps.

A series of experiments was designed to ascertain the factors which affected the survival of various stages of I. trianguliceps, to find the most suitable conditions for laboratory maintenance and to construct a life cycle scheme.

Humidities were maintained either by glycerol/water mixtures (Johnson, 1940) for high humidities and sulphuric acid/water mixtures (Buxton, 1931) for low humidities. The mixtures were placed in the bottom of a sterilized plastic container to a depth of $1\frac{1}{2}$ inch to maintain the required humidities. The tubes to contain the ticks were placed in the container four days before use so that humidity could equalise. The ticks were placed in tubes which were capped by 100μm nylon mesh screening-silk. The plastic containers were placed in incubators at 26°C, 18°C and 10°C. The activity of the ticks was observed every day and the day of death was recorded.

Low humidities were found to lower the period of survival greatly. At 30% R.H. unfed larvae survived at 26°C, 18°C and 10°C on average for 2.8, 3.4, and 6.8 days respectively while at 100% R.H. they survived at 26°C, 18°C and 10°C on average for 36, 46, and 89 days. At 90% R.H. unfed larvae survived for a period similar to that at 100% R.H. while at 50% R.H. the survival rate was much lower. Nymphs survived on average for a longer period, especially at low humidities.

Hence, it would appear that humidities between 90-100% R.H. are the most favourable for the survival of unfed stages while in combination with low temperatures the survival is much enhanced.

7.2. Engorgement of the stages of I. trianguliceps.

Three methods can be used to find the attachment periods of the stages of ticks; (a) the detachment from naturally feeding ticks from wild hosts, (b) by marking-recapturing of tick-infested wild hosts, and (c) the attachment period of laboratory fed ticks.

Both the capture and release capture methods are inaccurate since it is not known when the tick stages had attached to the host. However, it does give an indication of limits of the feeding period on natural hosts.

In the first method hosts were inspected for ticks immediately after capture and the positions on the hosts were recorded. The hosts were then placed in a respirator (Fig. 3) supplied with air of 100% R.H. The state of feeding of ticks at $\frac{1}{2}$-daily or daily intervals and the time of detachment was recorded (Table 42).

There was no evidence of a periodicity of detachment as reported by Balashov (1958) for I. persulcatus. There was no significant difference in feeding periods on M. agrestis, C. glareolus and A. sylvaticus.

Shrews were not investigated since there were no facilities for maintaining them in the laboratory.

The maximum larval feeding period was five days

TABLE 42: ENGORGEMENT OF I. TRIANGULICEPS ON WILD SMALL MAMMALS

	Days after Capture																		
	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0	13.0	14.0	15.0	16.0
Larvae	36	22	24	18	16	12	2	6	1											*
Nymphs	12	6	8	11	7	14	6	4	6	7	2	1								*
Adult ♀♀	2	1	3	1	1	2	-	1	-	3	1	2		1	1		2	1	1	*

4 Larvae failed to detach.

2 Nymphs failed to detach.

1 Adult ♀ detached at 21 days.

1 " " " at 28 days.

TABLE 43 : ENGORGEMENT OF I. TRIANGULICEPS ON LABORATORY ANIMALS*

	Days after Attachment												
	1.0	1.5	2.0	2.5	3.0	3.5	4.0	5.0	6.0	7.0	8.0	9.0	10.0
Larvae			1	8	16	6	2						
Nymphs					4	5	6	3					
Adult ♀♀									2	1	1	1	

* Inoculated with 0.2 ml. of Betsolan.

3 attached larvae failed to detach.

2 " nymphs " " "

1 " adult ♀ " " "

although some individuals failed to detach from the hosts. Over three-quarters of the total number of ticks had detached on the third day after capture (Table 42). The maximum period of nymphal attachment was observed to be 8 days while the majority had detached after 4 days. Adult female feeding periods appeared to be extremely variable and one adult did not detach for 28 days but the majority had detached by day 8, (Table 43).

7.3. Weights of stages of I. trianguliceps.

The weights of ticks give an indication of their blood meals size and the utilisation of the food supplies.

The eggs of I. trianguliceps were smaller than those of I. ricinus and I. hexagonus and were only $\frac{1}{2}$ to $\frac{1}{3}$ of the weight of eggs of I. hexagonus. This is not surprising since I. trianguliceps is one of the smallest members of the genus Ixodes (Table 44). Larvae weighed less than the eggs due to the loss of the eggs' shells and material used up during the formation of the embryo. The larvae were observed to be heaviest after hatching and decreased in weight during starvation. After attachment no blood was observed in the gut for the first day and a half and after this period the larvae became black or dark red due to the uptake of blood. The picture appeared to be similar to that of I. ricinus as reported by Sutton and Arthur (1962). No so-called "white" ticks, which according to Sutton and Arthur (1962) were formed by uptake of tissue fluid rather than blood were observed.

The weight of engorged larval stages were variable ranging from 0.193 mg. to 0.276 mg. This was an increase of 8 to 10 times that of the starved larval body weight.

The unfed nymphal stages weighed between 0.095 to 0.146 mg. depending on their gut contents (a variable amount of the larval feed remained in the gut after moulting). Fed nymphs weighed between 0.591 mg. and 0.735 mg. The feeding pattern was similar to that of larvae. However, only a five-fold increase in weight occurred with nymphs.

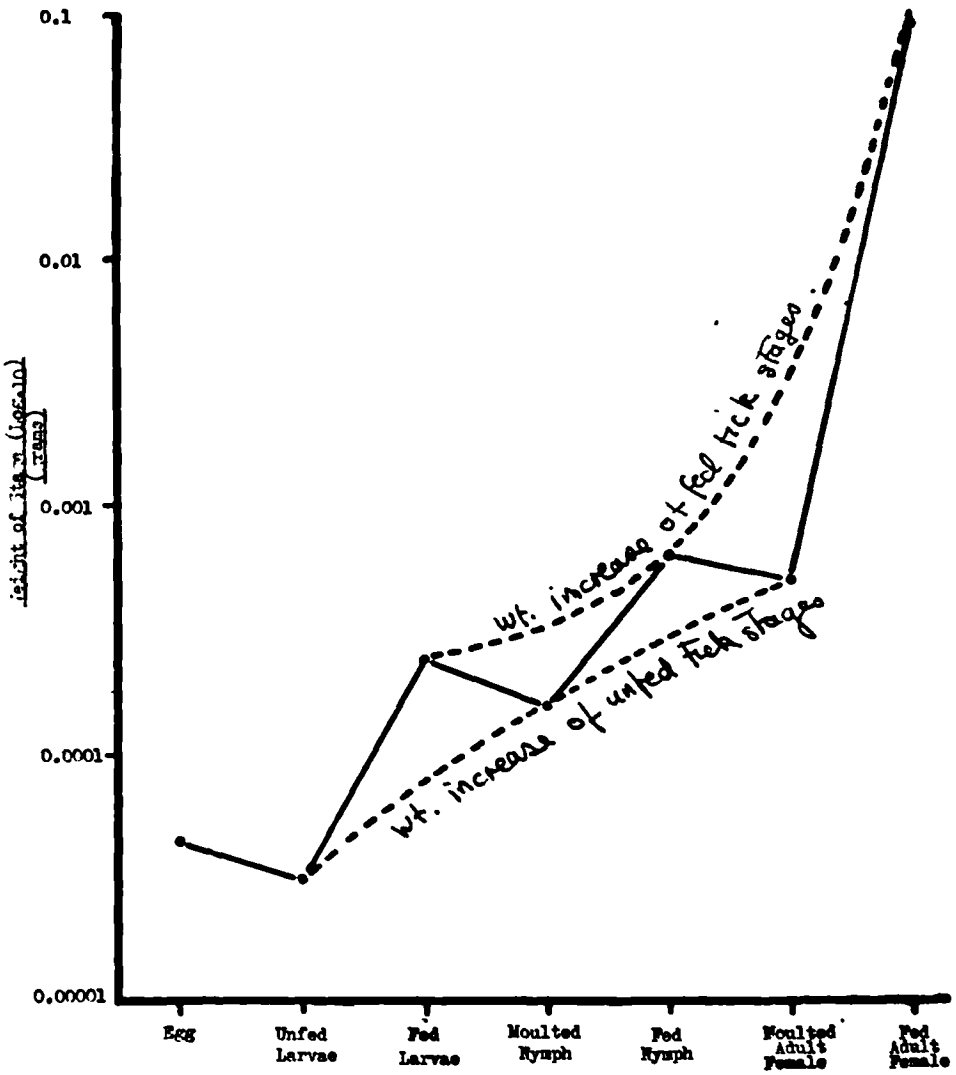
Unfed adult ♀♀ appeared to be heavier than unfed adult ♂♂ and ranged in weight from 0.481 mg. to 0.576 mg. while the males weighed between 0.405 and 0.527 mg. Fed adult ♀♀ showed a considerable range in weight on detachment from 35.81 mg. to 116.0 mg. This represented on average a 200-fold increase in weight during feeding (Fig. 91).

Therefore the blood meal obtained by an adult female from small mammals represents a high proportion of their blood volume. In small rodents the total blood volume is 7.7 ml./100 g. body weight (Wish et al, 1950). Many adult ♀♀ ticks may remove at least 100 mg. of blood which would represent 1/15 of the total blood of a 20 g. small mammal. If the adult fed on pigmy shrews this would represent approximately a quarter of the total blood volume. Newson (1962) reported that adult females I. trianguliceps caused a slight anaemia and reticulocytosis on C. glareolus.

TABLE 44: SHOWING THE WEIGHT OF STAGES OF I. TRIANGULICEPS

Egg mg.	Unfed Larvae mg.	Fed Larvae mg.	Unfed Nymphs mg.	Fed Nymphs mg.	Unfed ♀♀ mg.	Unfed ♂♂ mg.	Fed Adult ♀♀ mg.
0.045	0.031	0.276	0.096	0.670	0.563	0.405	73.94
0.048	0.034	0.235	0.099	0.730	0.526	0.510	35.81
0.039	0.026	0.222	0.112	0.690	0.576	0.467	110.0
0.039	0.039	0.252	0.123	0.735	0.495	0.527	102.0
0.047	0.032	0.263	0.110	0.652	0.486	0.472	99.0
0.049	0.029	0.216	0.136	0.578	0.562	0.543	89.0
0.052	0.035	0.234	0.091	0.762	0.529		101.0
0.048	0.031	0.246	0.146	0.735	0.476		114.0
0.044	0.035	0.193	0.123	0.656	0.532		106.0
0.041	0.036	0.265	0.114	0.732	0.481		93.0
0.053	0.029	0.210	0.106	0.672	0.492		89.0
0.045	0.034	0.220	0.102	0.651	0.537		95.0
0.043	0.029	0.199	0.116	0.591			98.0
0.041	0.037	0.273	0.135	0.683			103.0
0.039	0.028	0.245	0.142	0.720			116.0
0.048	0.031	0.206	0.995	0.735			87.0
0.047	0.031	0.234	0.113	0.632			102.0
0.046	0.032	0.256	0.132	0.652			94.0
0.051	0.034	0.245	0.121	0.672			
0.043	0.036	0.237	0.119	0.612			
Ave. 0.045	0.032	0.236	0.116	0.683	0.521	0.496	94.7

Fig. 91: Increase in Weight of Stages of I. triangulicens During Their Life Cycle



7.4. Laboratory feeding of ticks.

Investigations were made to improve the success of feeding ticks.

(a) Effect of temperature and length of starvation on the feeding of I. trianguliceps stages.

Newly hatched larvae were placed in tubes at 100% R.H. These tubes were placed in 22°C and 7°C incubators. At 22°C, it was observed that the success rate of feeding was increased with time. No ticks were observed to feed 7-14 days after hatching. However after 50 days 5/10 ticks successfully fed (Table 45). At 7°C, no ticks were observed to feed until day 30 after hatching and the success rate increased until day 50 when 10/2 ticks were fed (Table 45). Similar results were obtained with nymph and adult ticks. Therefore, the ticks had to be kept for a certain time after hatching before successful attachment and feeding would take place.

A similar effect was found by Lees, (1948) in the case of I. ricinus who suggested it was due to the physiological state of the ticks as judged by the response to host stimuli. Therefore, in high humidity dependent ticks, such as I. ricinus and I. trianguliceps the ticks have to attach to the host rapidly or they will die due to desiccation.

Experiments also demonstrated that hair restricted the attachment of I. trianguliceps probably due to desiccation produced before the tick can reach the skin. Therefore all hosts were shaved before ticks were fed on them.

(b) Effect of cortisone-like substances on the feeding of I. trianguliceps.

Hosts were inoculated intramuscularly with 0.2 ml . of Betasolan (see Section V 9) 2 days before feeding the ticks and 0.2 ml. after the first day of feeding. The attachment rates of ticks were not noted to differ between inoculated and control hosts. However, on control hosts, it was observed that a proportion of ticks failed to detach normally on the completion of feeding. When the animals were treated with Betasolan this phenomenon was not noted. The actual feeding period was observed to be shorter. The mechanism of this effect was not known but it would appear to reduce the host response to ticks.

(c) Conditions most favourable for maintenance of ticks in the laboratory.

By experimentation it was found the most favourable humidities to maintain I. trianguliceps, H. anatolicum, H. dromedarii, I. ricinus, and I. hexagonus were 100%, 80%, 90%, 100% and 90% R.H. respectively. The most satisfactory temperatures for each stage of the ticks is shown in Table 46. The completion of each stage was shortened by raising the temperature but this tended to lower the feeding success rate in many cases.

(d) Success rate of tick feeding during laboratory experiments.

The success rate of tick feeding is shown in Table 46. Rats appeared to be slightly better hosts for Hyalomma anatolicum and Ixodes hexagonus than mice. However mice appeared to be better hosts for I. trianguliceps than rats. The highest success rate using the method described was for Hyalomma anatolicum while the lowest success rates were for I. trianguliceps.

TABLE 45: SHOWING THE SUCCESS OF FEEDING OF I. TRIANGULICEPS (LARVAE) AFTER DIFFERENT PERIODS OF STARVATION

Temperature	Time After Hatching (Days)	No. of Ticks in Groups	No. Feeding Successfully
22	7	10	-
22	14	9	-
22	21	10	1
22	30	10	2
22	40	10	3
22	50	10	5
7	7	20	-
7	14	20	-
7	21	20	-
7	30	20	4
7	40	20	8
7	50	20	16
7	70	20	14
7	90	20	6

TABLE 46: CONDITIONS USED TO MAINTAIN TICKS IN THE LABORATORY

Tick Species	Humidity % R.H.	Hatching Temp. °C	Larvae Hardening Temp. °C	Larvae Moulting Temp. °C	Nymph Hardening Temp. °C	Nymph Moulting Temp. °C	Adult Hardening Temp. °C
Hyalomma anatolicum	80	28	22	28	22	28	22
Hyalomma dromedarii	90	28	22	28	22	28	22
Ixodes ricinus	100	22	15	22	15	22	15
Ixodes triangulicep	100	20	10-15	22	10-15	22	10-15
Ixodes hexagonus	90	22	15	22	15	22	15

TABLE 47: THE SUCCESS RATE OF TICK FEEDING IN LABORATORY EXPERIMENTS

Tick Species	Host	Percentage of Ticks Fed Successfully		
		Larvae	Nymphs	Adults
		% out of 1,000 (rats) or 500 (mice)	% out of 500 (rats) or 200 (mice)	% out of 30 (rats) or 10 (mice)
Hyalomma anqtolicum	Rats	77.5	73.6	
	Mice	75.4	73.0	80
Hyalomma dromedarii	Rats	75.5	74.4	80
Ixodes ricinus	Rats	71.1	57.0	75
Ixodes triangulíceps	Rats	57.7	60.4	60
	Mice	63.6	70.0	70
Ixodes hexagonus	Rats	73.8	70.0	75
	Mice	70.8	67.5	70

7.5.1. Life cycle of I. trianguliceps.

From data obtained on the attachment period of tick stages on hosts, it is apparent that ticks only spent a short period of their cycle parasitic on hosts (≏ 16 days). Hence by far the longest period of the life cycle must be spent on the ground where they had detached from their hosts. Unfortunately the majority of information obtained on the life cycle of I. trianguliceps in nature was obtained from seasonal infestation of hosts. This was not an accurate way of determining the life cycle since it was dependent on the nature of the small mammal populations as well as on the development of tick populations.

If the seasonal infestation of I. trianguliceps is combined with data obtained from laboratory experiments, two types of life cycle are evident (Table 48). Two peaks of adult female infestation were seen in the Spring or early Summer and the Autumn and the resultant life cycle produced by these two groups of adult ♀♀ would take different periods to complete; the Autumn female ticks probably overwinter in the pre-oviposition period which appeared to be a built in mechanism since it was extremely long even under conditions favourable for oviposition. However, if the peak occurred earlier, in September, it was possible that oviposition started in the Autumn. This would explain why resultant peaks of larvae were seen during June and July at Blean and during April at Silwood. During the Spring the rise of temperature would allow oviposition and hatching providing larvae for infestation between April and June. This appeared to be the longest period of the life cycle which lasted in nature 240-310 days. However, in the laboratory the same process took

TABLE 48: PERIOD TAKEN TO COMPLETE LIFE CYCLE OF I. TRIANGULICEPS UNDER DIFFERENT CONDITIONS AT 100% R.H.

	22°C	15°C	Time calculated from seasonal infestation	
			Spring Adults	Autumn Adults
Preoviposition period	21-29	37-46	120	
Oviposition period	1-26	1-32		240 Peak in April
Hatching period	30-40	40		310 Peak in June
Prefeeding period of larvae	25	25		
Feeding of larvae	3	3	210	100 Peak in June
Larval moulting period	26	35		90 Peak in Autumn
Prefeeding period of nymphs	30	30		
Nymph feeding period	4	4	120	
Nymph moulting period	32	50		
Adult prefeeding period	40	40		210 Peak in April
Adult feeding period	7-9	7-9		260 Peak in June
Total time to complete life cycle	219-265	274-314	≏ 450	≏ 600-610

on average 120 days at 15°C. This longer period could be due entirely to the lower temperatures in nature since the preoviposition period, oviposition and hatching and hardening of larvae were temperature dependent. However, as already stated, there appeared to be a diapause or some other restricting effect which prevented oviposition of late Autumn fed adults until the Spring.

The Spring and early Summer peaks of larvae presumably moulted to produce an Autumn peak of nymphs (Cotton & Watts, 1967) or if the peak occurred in April an overlap of nymphal peaks would produce an overall peak in June/July. It is rather surprising that the autumnal peak of nymphs reported by Cotton and Watts (1967) was much lower than the early Summer peak, since the early Summer larval peak was of a size similar to the autumnal peak of larvae and the resultant adult ♀♀ peak in the next Spring was similar in size to that in the Autumn.

The moulting period of larvae to nymphs appeared to take 90-100 days compared with 65 days at 15°C in the laboratory. The autumnal nymphs overwintered in a pre-moulting stage to produce adult ♀♀ in the Spring or early Summer. Moulting was also shown to be temperature dependent and occurred during the Spring rise in temperature. This process appeared to take 210-260 days in nature compared with 90 days at 15°C in laboratory experiments. Hence in this case the life cycle took something in the region of 600 days to complete. The Spring fed adult ♀♀ completed their life cycle during two warm periods compared with only 1 warm period in the case of Autumn fed adult ♀♀. The Spring or early Summer fed adult ♀♀ went through oviposition and hatching immediately and the larvae became available for attachment to hosts from the end of August to produce the autumnal peak of larvae. On average this process took 120 days in nature compared with

110 days at 15°C in the laboratory. These resulting engorged larvae overwintered in the premoulting state and moulted during the Spring rise in temperature - the nymphs infesting hosts from April onwards. This process took approximately 210 days compared with 65 days at 15°C in the laboratory. These nymphs moult into adults to produce the Autumn peak. This period in nature was approximately 120 days compared with 90 days in the laboratory at 15°C. Therefore in this case the life cycle only lasts 450 days.

Therefore there is an alternation between long and short life cycles depending on whether the female fed in the Autumn or Spring. This represents a passive reaction to environmental conditions since a first generation which fed in the Spring produced a second generation of females which fed in the Autumn.

This is unlike I. ricinus where the bimodal peaks may represent definite populations of ticks which react to light and temperature in different ways (Campbell, 1950).

In the case of I. trianguliceps there was an overlap between the peaks so two groups were not totally separated and it would be possible for one stage to transfer to the other type of life cycle depending on environmental or host conditions.

The majority of ticks were seen to feed during the warmer periods of the year especially nymphs and adult ♀♀. However larvae were also observed on hosts during the colder periods of the year which was difficult to explain since the survival of larvae was found to be lower than

that of nymphs and adult ♀♀. However the oviposition and hatching periods were the longest periods of the ticks' life cycle, and were variable, which could result in a dispersed occurrence of larvae. Also larvae were seen to remain more active during colder conditions than nymphs and adults and could be more easily activated by external conditions, i.e. hosts.

The males were found to have a similar seasonal activity to adult ♀♀ as reported by Cotton and Watts (1967). However, no seasonal peak in April was observed and the occurrence of the female was very sporadic and only two were seen crawling through the hair. This would suggest that copulation usually occurred on the ground as suggested by Arthur (1962).

7.5.2. Population dynamics and mortality of I. trianguliceps.

From data obtained from laboratory experiments and field investigations, some conclusion can be made on the population dynamics of I. trianguliceps in natural conditions.

Certain assumptions have to be made. These are the concept of a unit host, that is average animal in relation to tick infestation as calculated from small mammal capture throughout the year.

The numbers of each tick stage feeding on a unit host in a given period, i.e. a month, can be calculated:-

$$\frac{\text{Infestation rate x time}}{\text{Average feeding period of stage}}$$

The average engorgement was found to be 2.5 days in larvae, 3.5 in the nymphs and 7 days in adults. From these calculations it is obvious that infestation rate was an inaccurate index of tick turnover in a certain period. Therefore, in comparison with the infestation rate of adult ♀♀, larvae and nymphs show an under-estimation of ≏ 3x and ≏ 2x respectively. The number of larvae fed per unit host varied between 3.7 ticks to 13.4 ticks a month. Over the whole year a unit host would feed 83 larval ticks. The number of nymphs fed on a unit per month varied between 0.03 and 3.6 and the total number of nymphs fed on a unit host per year was 14.15. The number of adult ♀♀ fed per month varied between 0.04 and 0.65 and the unit host fed 2.5 adult ♀♀ per year. The total number of ticks fed by a unit host per year was 99.65.

Using these data some conclusions can be made on the mortality of stages. From laboratory maintenance experiments the adult ♀♀ laid approximately 1,000 eggs under favourable conditions. Hence as 2.5 adults fed on an average host assuming they fed to replenishment they should produce 2,500 eggs.

The mortality of stages of I. trianguliceps can be calculated from the following formula:-

$$\frac{\frac{dx_1}{dy_1} - \frac{dx_2}{dy_2}}{\frac{dx_1}{dy_2}} \times 100$$

$\frac{dx}{dy}$ = slope of curve (Fig. 92)

Hence the mortality of larvae developing from potential egg production is 96.6% while from larvae to nymphs the mortality is 82.2%. The mortality between nymphs and adult females was difficult because a certain number became males. If the sex ratio was 50% the mortality to adult female stage would be 45%. The overall mortality between eggs and adults appears to be in the region of 99%.

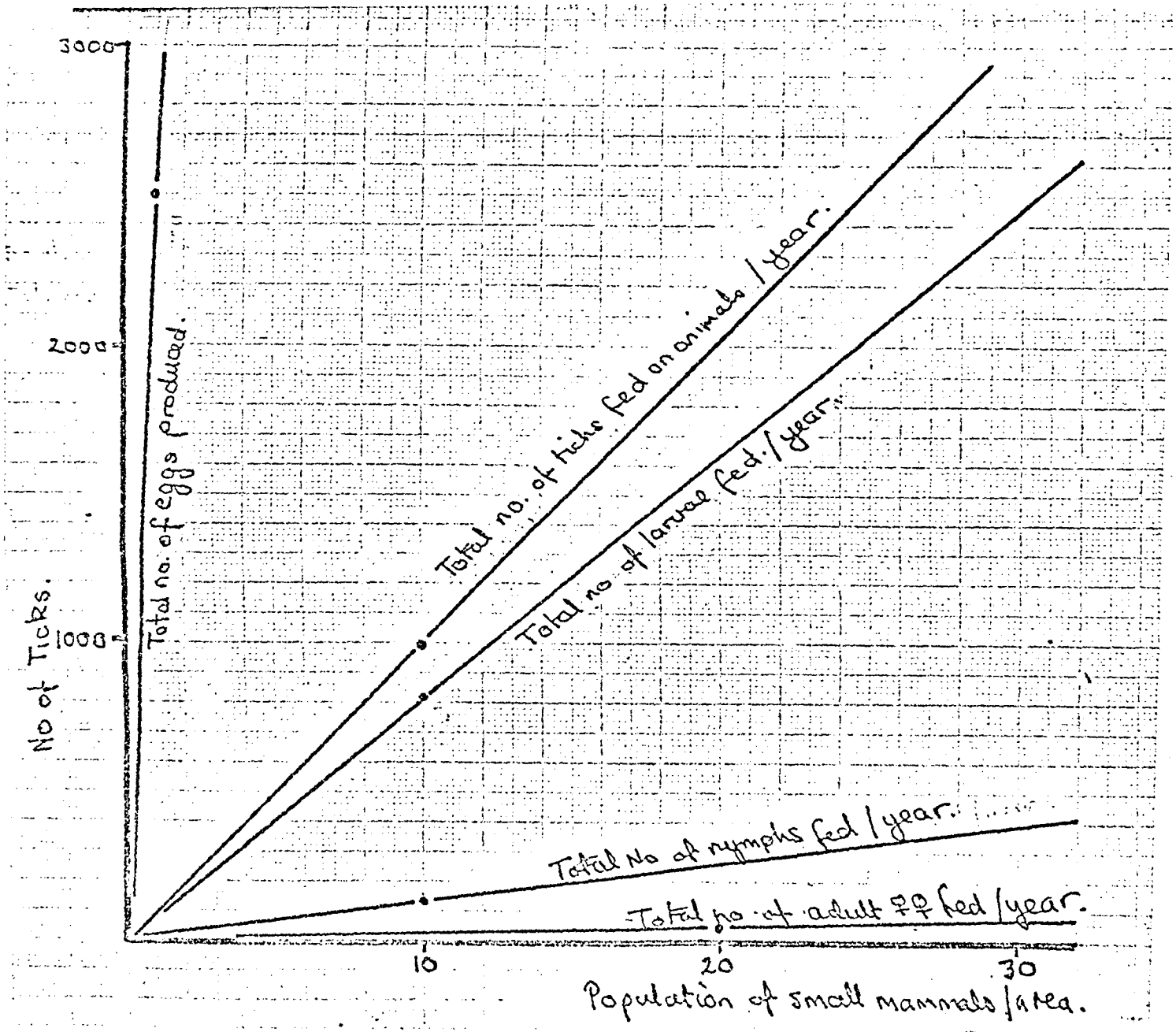


Fig. 92: A theoretical estimation of numbers of Ixodes trianguliceps stages fed by small mammal populations of different densities.

8. Transmission of B. microti and B. rodhaini.

The transmission of B. microti and B. rodhaini was studied to establish how they might be transmitted in nature and to reproduce such transmission in the laboratory.

8.1. Non-cyclical transmission.

Possible non-cyclical transmission, which could occur in nature, were considered to be mechanical, intrauterine and oral transmission.

8.1.1. Mechanical transmission.

Mechanical transmission, in this context, was considered for the transmission of Babesia spp. by means of the mouth parts of ectoparasites without cyclical change of the parasites; i.e. the ectoparasite's mouthparts act as a syringe.

(a) Survival of B. microti and B. rodhaini at different temperatures.

The survival of B. rodhaini and B. microti at different temperatures and in the bodies of different ectoparasites was studied.

Blood was obtained from infected mice and rats by cardiac puncture and was diluted with Alsever's solution until 10 million parasites per millilitre of solution was obtained. The solutions were stored under sterile conditions at 0°C, 10°C, 15°C and 25°C and were inoculated in 3 mice or rats, depending on the origin of the blood, 0, 5, 12, 24, 48 and 72 hours from the time of dilution. The production of an infection in the recipients was used

as an indicator of survival and the average time to the peak of parasitaemia was used as an index of the proportional survival of the parasites.

The results obtained are plotted in Fig.93. At 0°C B. rodhaini and B. microti in rat and mouse blood appeared to survive for between 48-72 hours while at higher temperatures the period of survival was reduced until at 25°C, the parasites only survived for 12-24 hours. A gradual loss of infectivity was apparent as indicated by the increase of time to reach the peak parasitaemia.

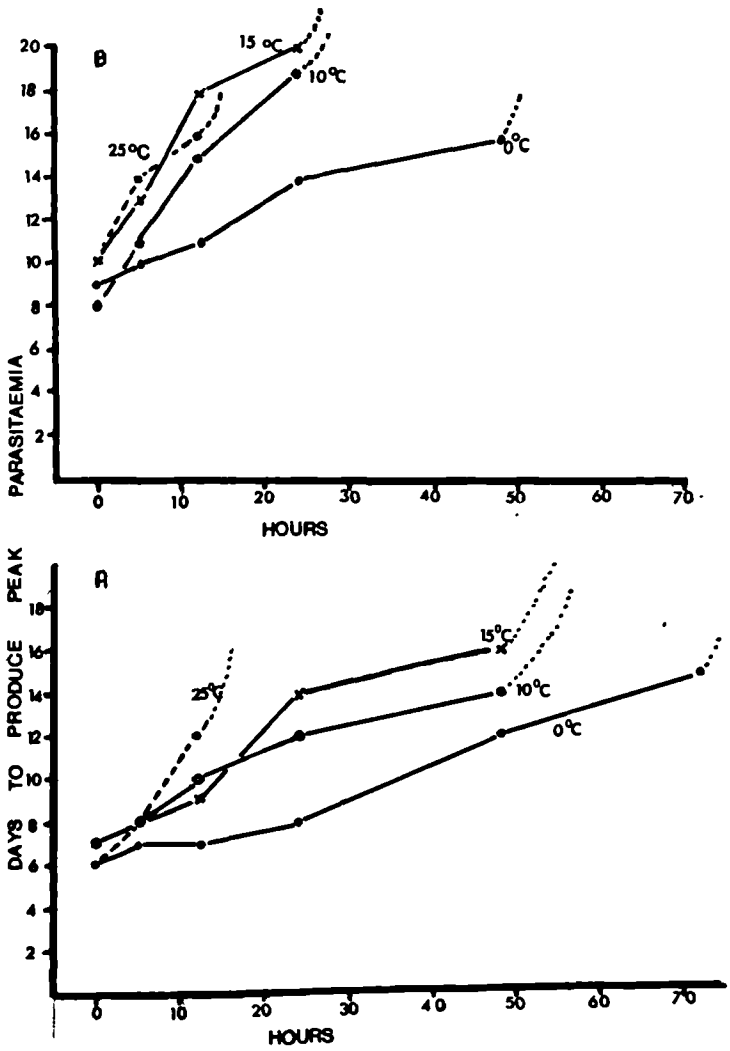
(b) Survival of B. microti and B. rodhaini in the body of ectoparasites at different temperatures.

Mechanical transmission can only be possible during the period the blood parasites remain infective in the body of the ectoparasites. To determine this period the survival of B. microti and B. rodhaini in the body of several species of ectoparasites was studied to assess the period that mechanical transmission is likely to occur in nature.

Ectoparasites (Cimex lectularius, Rhodnius prolixus and Aedes aegypti) were allowed to be engorged on B. microti and B. rodhaini infected rats and mice. (See Section III.3). The insects were stored at 0, 10, 15, and 25°C and were ground up in Alsever's solution in a mortar and pestle 0, 5, 12, 24, 42 and 72 hours after feeding on infected rodents. Amounts of the resultant solution containing approximately 10^6 parasites were inoculated into rats and mice. Infections, if any, were recorded and the time to reach peak parasitaemia was recorded.

Fig. 93: The survival of B. microti and B. rodhaini in Alsever's solution at different temperatures as judged by infectivity to rodents.

A. B. rodhaini in mice.
B. B. microti in rats.


B
15 °C
10°C
25°C
0°C
PARASITAEMIA
HOURS
A
15°C
25°C
10°C
0°C
DAYS TO PRODUCE PEAK
HOURS

The results obtained were similar to those recorded previously (a) (Fig. 9). There appeared to be little difference in the survival of blood parasites in these different insects. An increase in temperature significantly reduced the survival time of the parasites. B. microti and B. rodhaini will survive for up to 72 hours at 0°C but this decreased to between 12-24 hours at 25°C. Therefore, it would be theoretically possible for mechanical transmission to occur during the period of survival.

(c) Attempts to obtain mechanical transmission of B. microti and B. rodhaini.

For a blood sucking arthropod to be an efficient mechanical transmitter it must be able to feed several times over a short period (e.g. 24 hours) since at normal room temperatures, blood parasites have a short period of survival in the ectoparasites. During preliminary feeding experiments, Aedes aegypti was found to be unsuitable since these mosquitoes failed to feed several times. However, Cimex lectularius and Rhodnius prolixus were potentially good mechanical transmitters as they could be interrupted several times during feeding.

Before the commencement of the mechanical transmission, the Cimex and Rhodnius stock used was starved for 2 weeks to ensure that full blood meals would be taken. Rhodnius could feed on rodents for over 10 minutes and during this period they could be interrupted 4 times. Cimex blood meals were much smaller in volume and were ingested in a shorter period. Both rats and mice were shaved before being used in these experiments.

The blood sucking arthropods were placed in tubes covered with muslin tops. The experimental rodents, which

had previously been anaesthetised by Nembutal, were placed over the top of the tubes so that their bellies were in contact with the muslin tops. The rodents infected with high parasitaemias (40%) of B. microti and B. rodhaini were placed on the tubes and then replaced after the majority of the blood sucking anthropods had started feeding with non-infected rodents. This was repeated until all the anthropods had been fully replenished. This process was repeated for three days using clean stock every day. 10 Rhodnius and 20 Cimex were used in each tube.

No infections were observed to develop in any of the mice or rats which suggested that no mechanical transmission occurred.

8.1.2. Intra-uterine transmission.

The occurrence of intra-uterine transmission in the genus Babesia has been reviewed by Enigk (1942) and Neitz (1956). Neitz (1956) assumed that injuries to the placental blood vessels paved the way for piroplasms to migrate from mother to foetus. However, according to Neitz, this form of transmission was rare.

The gestation period of mice and rats is approximately 21 days. In experiments to ascertain whether intra-uterine transmission was prevalent in piroplasm infections of rodents, animals were infected with B. rodhaini or B. microti 2 weeks before and during pregnancy. The resultant offspring were inspected for piroplasm infections at 3 day intervals up to 32 days after birth. However, no infections were detected in the offspring which suggested that intra-uterine transmission did not occur.

8.1.3. Oral transmission.

Oral transmission is possible in natural environments due to cannabalism which has been observed in both wild and laboratory populations (Clarke, 1951, Section IV.4). If infected blood organs or engorged ectoparasites are eaten, it is possible that blood parasites enter through the walls of the enteric tract. This phenomenon has been reported for malarial parasites by Shortt and Menon (1940), Young (1941) and Satya Prakash (1956) who fed infected blood to animals. However, no reports have been made on oral transmission in the genus Babesia.

(a) Infections produced by whole infected blood.

Groups of mice and rats were starved for 12 hours. Blood was obtained from rats highly infected with B. microti and mice highly infected with B. rodhaini (40% parasitaemia). Groups of 5 mice or rats were fed with 0.2, 0.4, 0.6 and 1.0 ml. of whole heparinised blood using a dropping pipette.

Four out of twenty rats fed with B. microti infected blood developed infections (Table 49). The chances of infection appeared to increase with the increase of volume of blood fed. Similarly, two out of twenty mice fed with B. rodhaini became infected (Table 49). Infections were only observed to develop in the 0.6 and 1.0 ml. groups.

TABLE 49: ORAL TRANSMISSION OF B. MICROTI AND B. RODHAINI IN INFECTED BLOOD

Species of Blood Parasite	Volume of Blood Fed	Recipient	Infections
B. rodhaini	0.2 ml.	Mice	0/5
"	0.4 ml.	"	0/5
"	0.6 ml.	"	1/5
"	1.0 ml.	"	1/5
B. microti	0.2 ml.	Rats	1/5
"	0.4 ml.	"	0
"	0.6 ml.	"	1/5
"	1.0 ml.	"	2/5

(b) Infections produced by infected viscera.

Sergent and Poncet (1957) reported the transmission of Plasmodium berghei after feeding the infected viscera of mice. This is a possible mechanism of transmission of small mammal piroplasms as cannabalism has been reported in wild and laboratory populations (Clarke, 1951, Section V.8).

In this experiment, rats highly infected with B. rodhaini and B. microti were killed and liver and spleens were dissected out. These organs were then fed to clean rats which had been starved for 12 hours.

Observations were made for any developing parasitaemia 3 to 32 days after feeding of the organs. One rat out of of ten which had been fed with B. microti infected viscera developed an infection while two out of ten rats, fed with B. rodhaini infected organs, developed parasitaemias.

(c) Infections produced by the ingestion of ectoparasites.

Small mammals tend to consume their ectoparasites during cleaning procedures or reactions to irritation caused by them. Milne (1949) describes the consumption of I. ricinus by voles, Arthur (1953) I. hexagonus by hedgehogs, and in the present study it has been observed in wild and laboratory animals (Section V.7). These ticks and some other blood sucking arthropods, contain large quantities of blood, which may remain infective for some time (Section V.8).

Ten adult Hyalomma anatolicum females which had just engorged with B. microti infected blood were fed to rats. The rats consumed the ticks with great rapidity. Two out of ten rats became infected with B. microti so demonstrating that oral infection can be produced by eating

ectoparasites.

8.2. Cyclical transmission

Neither B. microti nor B. rodhaini have been previously cyclically transmitted in the laboratory. Aeshlimann and Suter (1965) reported that they failed to produce cyclic transmission of B. rodhaini although they attempted it using several species of tropical ticks. They concluded that they either did not use the correct species of tick or that piroplasms had lost their ability to infect ticks due to continual needle passage in laboratory rodents. Therefore, in the present study, all stages of five species of tick were used in attempts to transmit B. microti and B. rodhaini.

From field observations, it was felt that I. trianguliceps was the most likely vector of B. microti since it was the only tick species to be found concurrently with B. microti infections in wild small mammal populations (see Section IV.4). I. trianguliceps was associated with small mammal populations in all its stages. There was no circumstantial evidence that other British ticks such as I. hexogonus and I. ricinus transmitted B. microti since they were found to be restricted in their occurrence on small mammals (Section IV.4).

British ticks have been found to be difficult to maintain in the laboratory due to a long life-cycle, and sensitivity to humidity and temperature. Hence, in order to transmit B. microti in the laboratory and to establish the host range of the vector, transmission was attempted using tropical ticks as well as indigenous British species.

The investigations on cyclical transmission of B. microti and B. rodhaini were designed to determine

the species and stages of tick which would transmit them and the nature of cyclical development within the ticks.

Strain of parasites.

The strain of blood parasites used had to be considered since there was some evidence that piroplasms may lose their ability to infect ticks after prolonged needle passage. (Aeschlimann and Suter, 1965; Riek, 1968).

Therefore, in the case of B. microti in rats, a recently isolated strain was used (S_4 see Section V.6), but this was not possible with B. microti in mice since strains had to be adapted through rats (S_2 see V.6). B. rodhaini had been isolated for 19 years so that it must have been passaged several hundred times (over 1000 times, Beveridge, 1969).

Rodent hosts.

The nature of rodent host affected the level of parasitaemia produced by the piroplasms and the number of ticks which could be fed on them. Rats had an advantage over mice since more ticks could be fed on them but a higher parasitaemia occurred in mice with B. rodhaini and B. microti. It was decided to use rats in the main transmission experiments and make replicates using mice in case the infectivity of piroplasms to ticks differed.

Experimental protocol of transmission experiment.

General methods used for feeding ticks on rodents are described in Sections III, V.7.

The general considerations for experimental transmission of piroplasms were the following:-

(1) ticks must be applied to infected hosts so that an adequate

parasitaemia (10%) would occur in the blood meal of the ticks;

(2) each possible type of transmission (interstadial and transovarian) must be tested;

(3) susceptible rodents must be used in transmission experiments;

(4) the same procedure must be carried out in transmission experiments using different species of ticks so that an equal opportunity would be given to each species.

The experimental design decided on is shown in Table 50. The transmission types studied were from larvae to nymphs, larvae to adults, nymphs to adults, nymphs to the larvae of next generation and adults to the larvae of the next generation. It was found possible to feed successfully 100 larvae, 50 nymphs and 2 ♂♂ ♀♀ on each rat and these numbers were applied to each animal during transmission experiments. For each type of transmission, 5 rats were used. In the case of mice (Table 51) only 50 larvae, 20 nymphs and one adult female could be fed. Five species of ticks were used for these experiments (Hyalomma anatolicum, H. dromedarii, Ixodes ricinus, I. trianguliceps and I. hexagonus). These species were chosen for their availability and likelihood of the piroplasm's transmission.

The methods of tick feeding and maintenance are described in Sections III and V.8. Typical success of tick feeding is shown in Table 4.

TABLE 50: EXPERIMENTAL PROTOCOL FOR TICK TRANSMISSION EXPERIMENTS USING RATS

Tick Species	Transmission Stage of Ticks	Blood Parasites	No. of Ticks Used	No. of Replicates
H. dromedarii	larvae to nymphs	B. microti	100 larvae 50 nymphs	5
"	larvae to adults	"	100 larvae 4 adults (2♂♂ 2♀♀)	5
"	nymphs to adults	"	50 nymphs 4 adults (2♂♂ 2♀♀)	5
"	nymphs to larvae F_i	"	50 nymphs 100 larvae	5
"	adults to larvae F_i	"	4 adults 100 larvae	5
"	larvae to nymphs	B. rodhaini	100 larvae 50 nymphs	5
"	larvae to adults	"	100 larvae 4 adults (2♂♂ 2♀♀)	5
"	nymphs to adults	"	50 nymphs 4 adults (2♂♂ 2♀♀)	5
"	nymphs to larvae Fi	"	50 nymphs 100 larvae	5
"	adults to larvae F_i	"	4 adults (2♂♂ 2♀♀) 100 larvae	5

TABLE 51: EXPERIMENTAL PROTOCOL FOR TICK TRANSMISSION EXPERIMENTS USING MICE

Tick Species	Transmission Stage of Ticks	Blood Parasites	No. of Ticks Used	No. of Replicates
H. anatolicum	larvae to nymphs	B. microti	50 larvae 20 nymphs	5
"	larvae to adult	"	50 larvae 1 ♀	5
"	nymph to adult	"	20 nymphs 1 ♀	5
"	nymphs to larvae F_i	"	20 nymphs 50 larvae	5
"	adults to larvae F_i	"	1 ♀ 50 larvae	5
"	larvae to nymphs	B. rodhaini	50 larvae 20 nymphs	5
"	larvae to adults	"	50 larvae 1 ♀	5
"	nymphs to adults	"	20 nymphs 1 ♀	5
"	nymphs to larvae F_i	"	20 nymphs 50 larvae	5
"	adults to larvae F_i	"	1 ♀ 50 larvae	5

Transmission of B. microti between rats.

Transmission of B. microti between rats was only obtained between the larval and nymph stage using I. trianguliceps (Table 52). All other stages and species of ticks produced negative results. Four out of the five replicates of transmission between larval and nymph stages of I. trianguliceps produced positive results (Table 52). The typical course of transmission is shown in Fig. 95 . Therefore it would appear that B. microti was only transmitted between the larval and nymph stages by the tick I. trianguliceps.

Transmission of B. microti between mice.

Similar results were obtained for the transmission of B. microti between mice (Table 52). All types of transmission except between larval and nymphal stages of I. trianguliceps proved negative (Table 52). Only two out of five replicates were positive in this case. A typical course of transmission is shown in Fig. 96 .

Transmission of B. rodhaini between rats.

All transmission experiments of B. rodhaini between rats failed to produce positive results (Table 52).

Transmission of B. rodhaini between mice.

All transmission experiments of B. rodhaini between mice failed to produce positive results (Table 52).

TABLE 52: RESULTS OF TICK TRANSMISSION EXPERIMENTS

RATS AS RECIPIENTS

Tick Species	Blood Parasite	Positive Transmissions	No. of Replicates Positive
H. dromedarii	B. microti	none	-
	B. rodhaini	none	-
H. anatolicum	B. microti	none	-
	B. rodhaini	none	-
I. triangulicaps	B. microti	Larvae-Nymphs	4/5
	B. rodhaini	none	-
I. hexagonus	B. microti	none	-
	B. rodhaini	none	-
I. ricinus	B. microti	none	-
	B. rodhaini	none	-

MICE AS RECIPIENTS

Tick Species	Blood Parasite	Positive Transmissions	No. of Replicates Positive
H. anatolicum	B. microti	none	-
	B. rodhaini	none	-
I. triangulicaps	B. microti	Larvae-Nymphs	2/5
	B. rodhaini	none	-
I. hexagonus	B. microti	none	-
	B. rodhaini	none	-

Fig. 95: A typical course of transmission of *B. microti* by *I. triangulíceps* larvae and nymphs between rats.

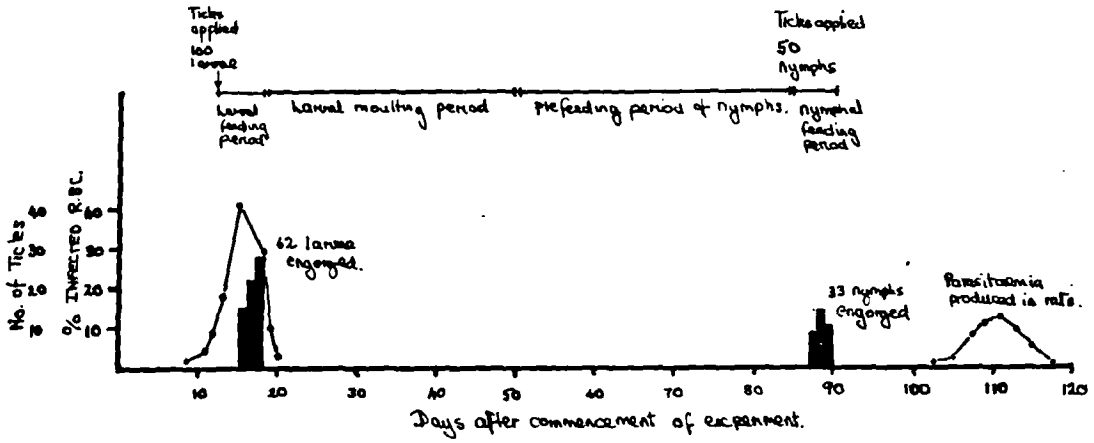
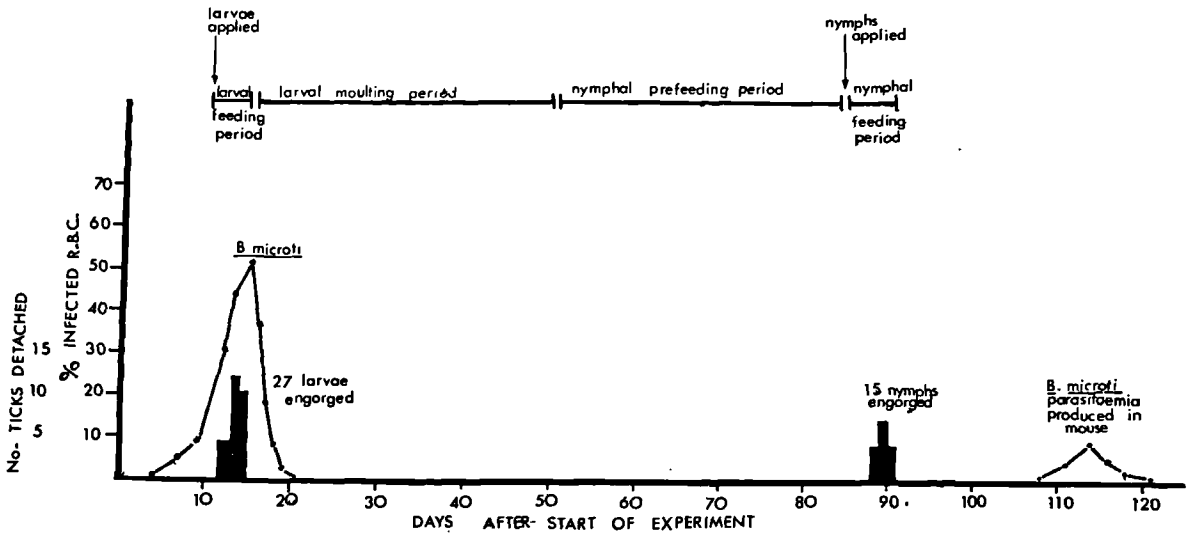

Ticks applied 100 larvae
Ticks applied 50 nymphs
Larval feeding period
Larval moulting period
Prefeeding period of nymphs.
Nymphal feeding period
No. of Ticks
% INFECTED R.B.C.
62 larvae engorged.
33 nymphs engorged
Parasitaemia produced in rats.
Days after commencement of experiment.

Fig. 96: A typical course of transmission of B. microti by I. trianguliceps larvae and nymphs between mice.


larvae applied
nymphs applied
larval feeding period
larval moulting period
nymphal prefeeding period
nymphal feeding period
B microti
27 larvae engorged
15 nymphs engorged
B. microti parasitaemia produced in mouse
% INFECTED R.B.C.
No. TICKS DETACHED
DAYS AFTER START OF EXPERIMENT

The course of infection produced by cyclic transmission.

The parasitaemia produced in experimental transmission animals is shown in Figs. 96b and 96c. The period of tick feeding in relation to parasitaemia is shown. It is noticeable that the original needle passaged parasitaemia was much higher than the parasitaemia produced in animals infected by ticks.

The prepatent period of B. microti infections transmitted by 15-40 nymphal I. trianguliceps was 10-15 days compared with 3 - 5 days in the normal needle passage infections. This type of parasitaemia is very similar to that produced by needle passage of B. microti which had been recently isolated from wild animals. The cyclically transmitted parasite appeared to grow much slower than the needle passage parasite. The maximum parasitaemia produced in rats or mice was 10-16% between 18-25 days after application of ticks.

The period of infectivity of I. trianguliceps nymphs infected with B. microti.

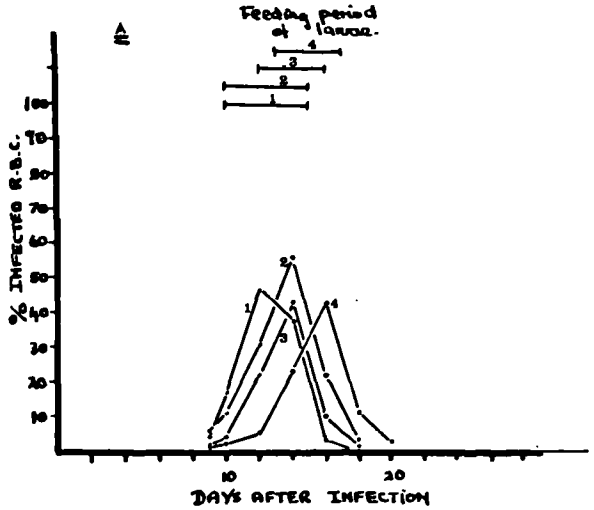
Nymphal ticks which had been fed previously on B. microti infected rats as larvae were applied to susceptible rats. They were observed to attach and 10 ticks were removed from rats at 5, 12, 24, 48, 72 and 96 hours after attachment. They were ground up in phosphate saline buffered at pH 7.2 in a mortar and pestle. Equal amounts of the resultant supernant was inoculated intraperitoneally in 2 rats. The inoculated rats were observed daily for a developing parasitaemia until day 32.

Infective parasites were evident in the ticks between 24 and 72 hours after the commencement of feeding of the

Fig. 96B: The parasitaemias of B. microti used to infect Ixodes triangulíceps larvae and the parasitaemias produced in rats by the corresponding nymphs.

A Infection of larvae.

B. Infection produced by nymphs.


A
Feeding period of larvae.
4
3
2
1
% INFECTED R.B.C.
10
20
DAYS AFTER INFECTION

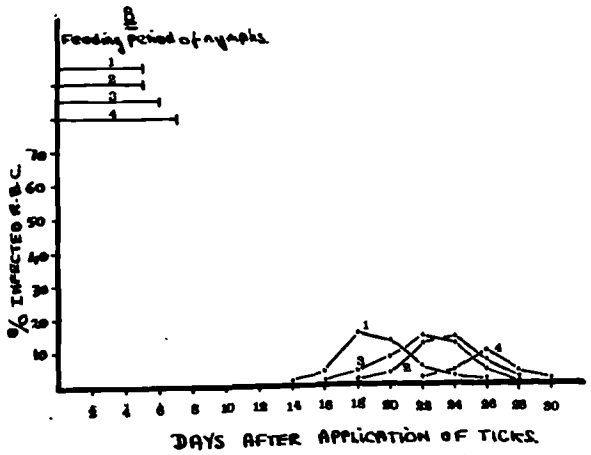
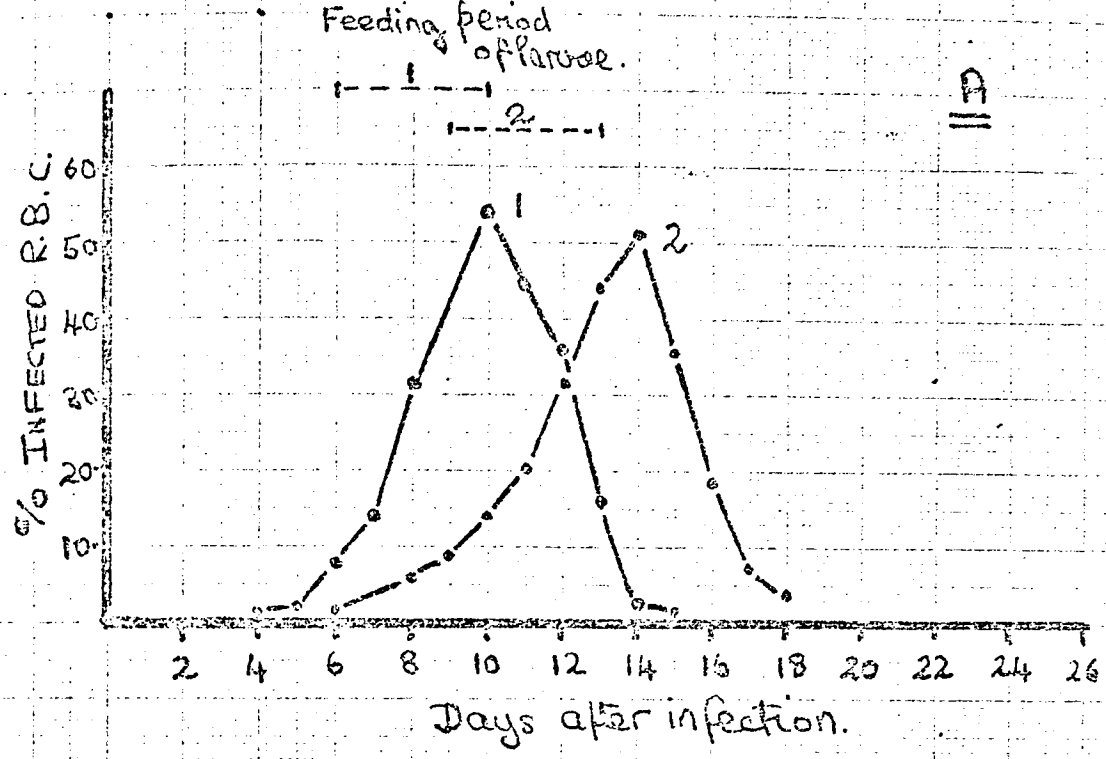

B
Feeding period of nymphs.
1
2
3
4
% INFECTED R.B.C.
DAYS AFTER APPLICATION OF TICKS.

Fig. 96C: The parasitaemia of B. microti used to infect Ixodes trianguliceps larvae and the parasitaemias produced in mice by the corresponding nymphs.

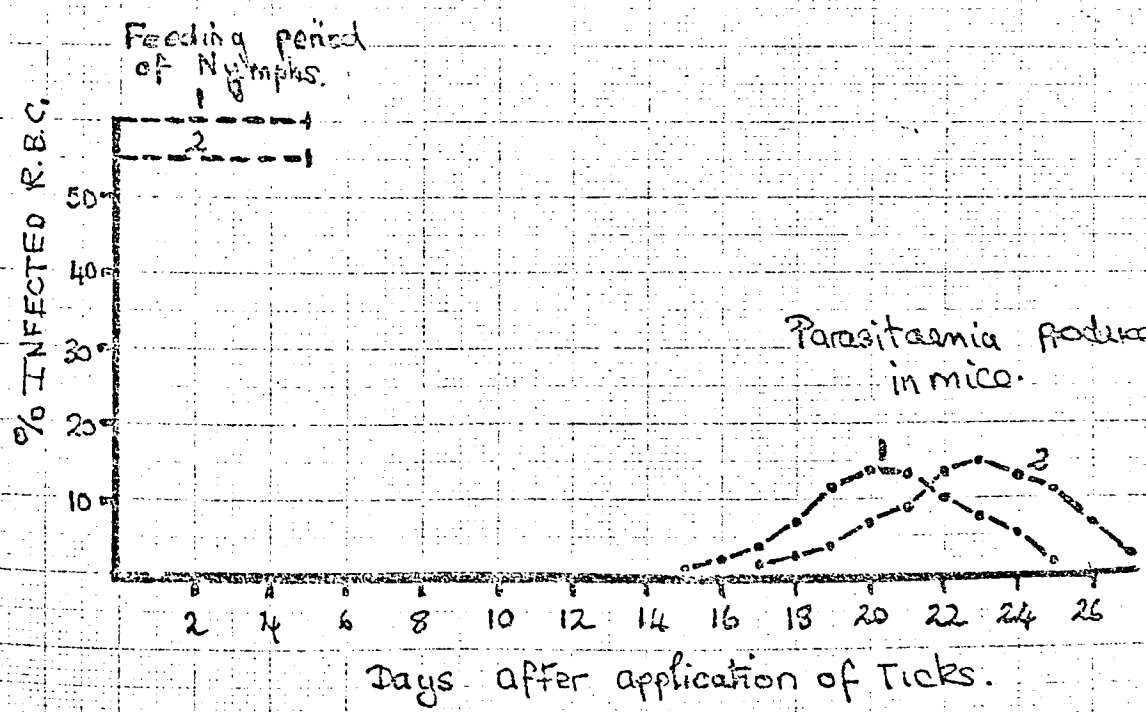
A Infection of larvae

B Infection produced by nymphs.

INFECTION OF TICKS.



TRANSMISSION OF B. MICROTI



nymphs. No infection developed at 5, 12, or 96 hours which suggested that parasites underwent a co-ordinated development within the ticks and that they were inoculated into the rodents between 24 and 72 hours after they started to feed.

Infection rate of a batch of nymphal I. triangulíceps.

The infection rate of ticks obviously affects the efficiency of transmission of piroplasms in both natural and laboratory conditions. This is especially applicable in this case since I. triangulíceps only occurs on hosts in small numbers (Section IV.4).

Individual nymphs were removed from rats 48-72 hours after attachment (the period of maximum infection as indicated by the previous experiment). These ticks were ground up in phosphate saline buffer at pH 7.2 in a mortar and pestle. The resultant supernant was inoculated in a single susceptible rat on each occasion. The blood smears were taken at daily intervals until day 30.

Nine out of 20 ticks proved to be infective for rats. Hence, it would appear that the infection rate of ticks in this batch was nearly 50% and that a one tick challenge using this batch of ticks would produce infection in nearly 50% of the cases.

The survival of infective blood forms of B. microti and B. rodhaini in different species of ticks.

The survival of B. microti and B. rodhaini was studied in ticks to determine changes in the parasite population in the tick gut, and to see if it differed according to whether ticks became infected or not.

From the evidence obtained from previous transmission experiments it was decided to use larval ticks of two species - H. anatolicum and I. trianguliceps. These larvae were fed on rats highly infected with B. microti and mice highly infected with B. rodhaini. Larvae were taken after engorgement and placed in groups which would provide about 5 million piroplasms in the blood. They were stored at 0, 10, 15, 25 and 32°C and ground up in Alsever's solution and inoculated in mice and rats at 0, 5, 12, 24, 48, and 72 hour intervals.

The results are shown in Fig. 97 . B. rodhaini appeared to survive slightly longer in larval I. trianguliceps than larval H. anatolicum while B. microti survived for similar periods in both I. trianguliceps larvae and H. anatolicum larvae.

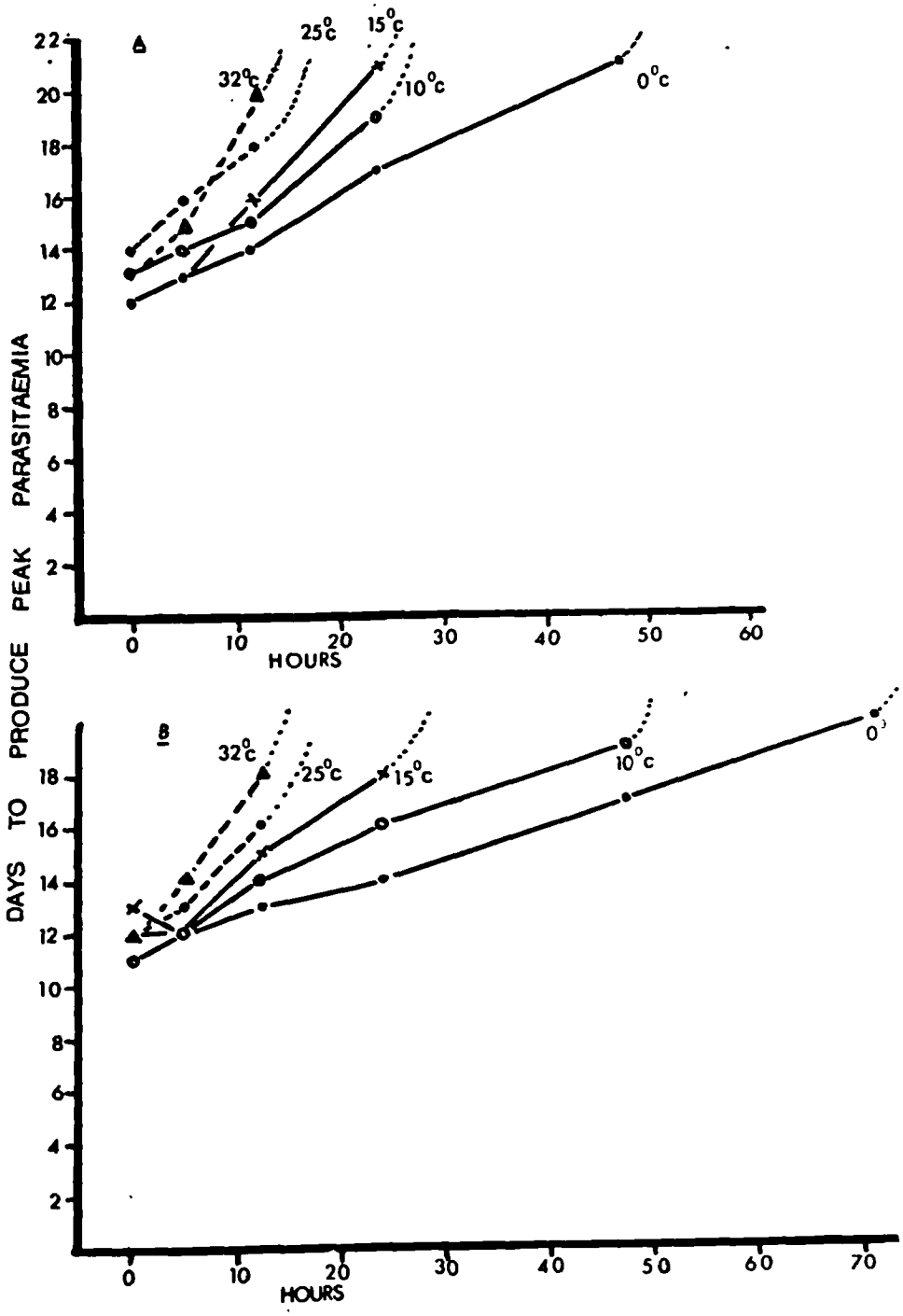
8.2.2. Development of piroplasms in ticks.

The nature of tick feeding has been studied in Ixodes ricinus by Hughes (1954) and Sutton and Arthur (1962) and reviewed by Arthur (1962). They found that ticks do not take in blood continuously and that there were several distinct stages during feeding. The ticks attach and appear to ingest fluids other than blood and in many cases, blood may be taken up during the last 24 hours of attachment only. Similar feeding features have been found in other genera of hard ticks (Snow, 1967).

Sutton and Arthur (1962) described white ticks which appeared to have engorged mostly on tissue fluids. They claim that the ratios of blood to tissue fluids in the blood meal varied considerably. In the present study no so-called white ticks were observed in either naturally or laboratory fed I. trianguliceps but have been observed in other species (H. anatolicum and H. dromedarii)

Fig. 97: The survival of B. microti in Hyalomma anatolicum and Ixodes trianguliceps at different temperatures as judged by infectivity to rats.

A. B. microti in H. anatolicum.
B. B. microti in I. trianguliceps.


A
32°c
25°c
15°c
10°c
0°c
22
20
18
16
14
12
10
8
6
4
2
0
10
20
30
40
50
60
HOURS
DAYS TO PRODUCE PEAK PARASITAEMIA
B
32°c
25°c
15°c
10°c
0°
18
16
14
12
10
8
6
4
2
0
10
20
30
40
50
60
70
HOURS

especially when the ticks were overcrowded.

In I. trianguliceps larvae, blood cells were observed in the gut, 24 hours after attachment only and rapid engorgement was observed after 48 hours. A similar situation was noted in I. trianguliceps nymphs although rapid uptake of blood was noted only after 72 hours. The adult females appeared to take a longer period to imbibe blood.

Larval I. trianguliceps increase approximately 0.20 mg. in weight during feeding while nymphs ingested about 0.55 mg. in comparison with adult females which imbibe 94 mg. of blood plus tissue fluids. However, Lees (1946) has shown I. ricinus loses great amounts of water during attachment to hosts. Hence, the volume of the blood ingested may be twice as much as the equivalent volume of the weight increase of the tick during feeding. In order to obtain a rough estimation of the number of parasites ingested by larval I. trianguliceps during the cyclical transmission of B. microti certain assumptions had to be made. It was apparent that the larval I. trianguliceps ingest between 0.2 μl to 0.4μl of blood and it had to be assumed that the blood meals consisted of only blood and the R.B.C. count was 7×10^6 R.B.C./μl. The number of parasites ingested by larvae can be estimated by the following formula.

$$\text{No. of parasites ingested} = \frac{\text{Volume of blood ingested } (\mu l) \times \text{R.B.C. count } (/\mu l)}{\text{Parasitaemia } (\%)}$$

The estimated number of parasites ingested is shown in Table 53.

TABLE 53: ESTIMATION OF THE NUMBER OF PARASITES (B. MICROTI) INGESTED BY LARVAL I. TRIANGULICEPS

Level of Parasitaemia in Rodents	No. of Ingested Parasites in Larval I. trianguliceps
0.001	2.3×10
0.01	2.3×10^2
0.1	2.3×10^3
1.0	2.3×10^4
10.0	2.3×10^5
100.0	2.3×10^6

The average level of parasitaemia found in wild populations was 0.05-0.5% (Section IV.4) so that an average larval I. trianguliceps would ingest approximately 1,150-11,500 parasites.

The conditions which occur in the tick's guts are probably very important for the infection of these hosts. The stomach of ticks consists of a small sac from which a number of blind ending pouches or diverticula originate (Fig. 98). In I. trianguliceps, the stomach leads into two branched, forwardly directed caeca and four unbranched caeca projecting backwards and lying side by side. The median pair of posterior caeca are larger than the lateral ones (Fig. 98). The cells of the mid-gut and diverticula vary in shape; some are small with basal nuclei and uniform non-vaculated cytoplasm while some are cuboidal, clavate, pedunculate or pyramidal with reticulated cytoplasm, frequently containing eosinophilic granules and large vesicular nuclei (Fig. 99). The epithelial cells of the gut lie on a basal membrane (Fig. 99).

Certain changes occur during the feeding period of the tick. A comparison of the gut epithelium during and after feeding is shown in Fig. 100. The most obvious change in the gut is its distension due to the uptake of blood. The basal membrane appears to become greatly extended so that the gut epithelial cells do not continually cover it. Certain gut epithelial cells are liberated into the lumen (Fig. 100 and Hughes, 1954). Metabolic products are seen to develop in these epithelial cells and are in many cases liberated into the gut lumen (Fig. 101). The blood meal becomes rapidly haemolysed after its uptake but certain red blood corpuscles remain non-haemolysed five days after detachment of the ticks from the hosts.

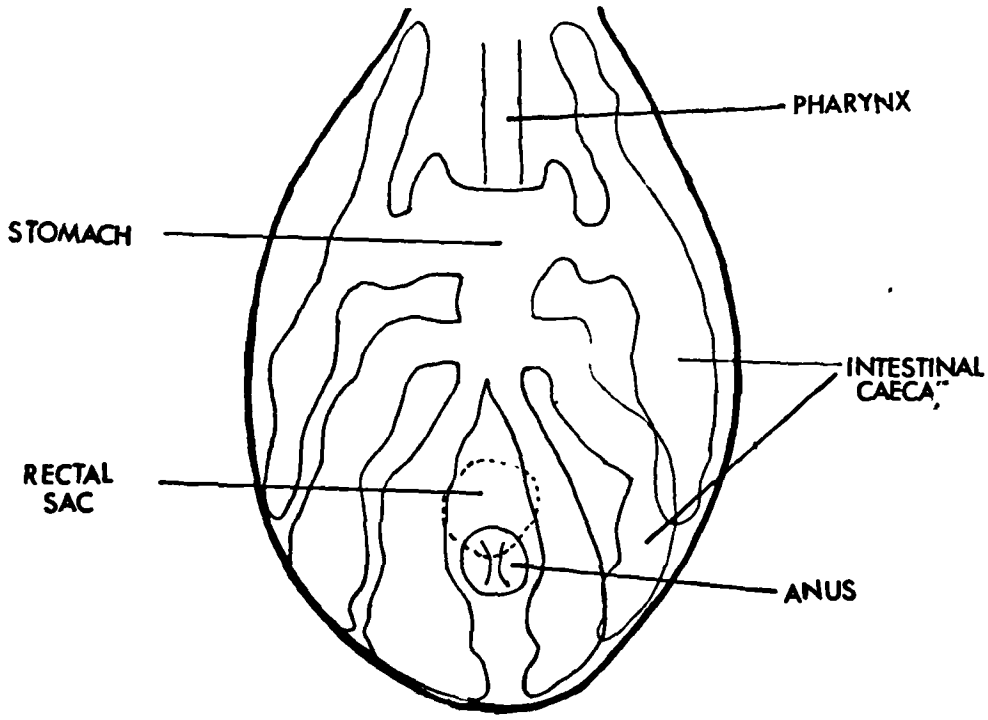


FIG. 98 DIAGRAM OF THE GUT OF LARVAL IXODES TRIANGULICEPS (x45).

Fig. 99: Transverse section of larval I. triangulíceps showing changes in the gut during feeding. (650×).

A. 24 hours after attachment.

B. 72 hours after attachment.

1. Vertical muscle bands.
2. Ingested bloodmeal (contracted during fixing).
3. Basal membrane of gut caecae.
4. Cuticle.
5. Sub-cuticular epithelium.
6. Gut epithelium cells liberated into gut lumen.
7. Metabolic granules (eosinophilic).
8. Salivary gland ducts.
9. Malphighian tubules.
10. Sub-cuticular cells.
11. Type II salivary gland (see Chinery, 1965).
12. Type III, salivary gland (see Chinery, 1965).

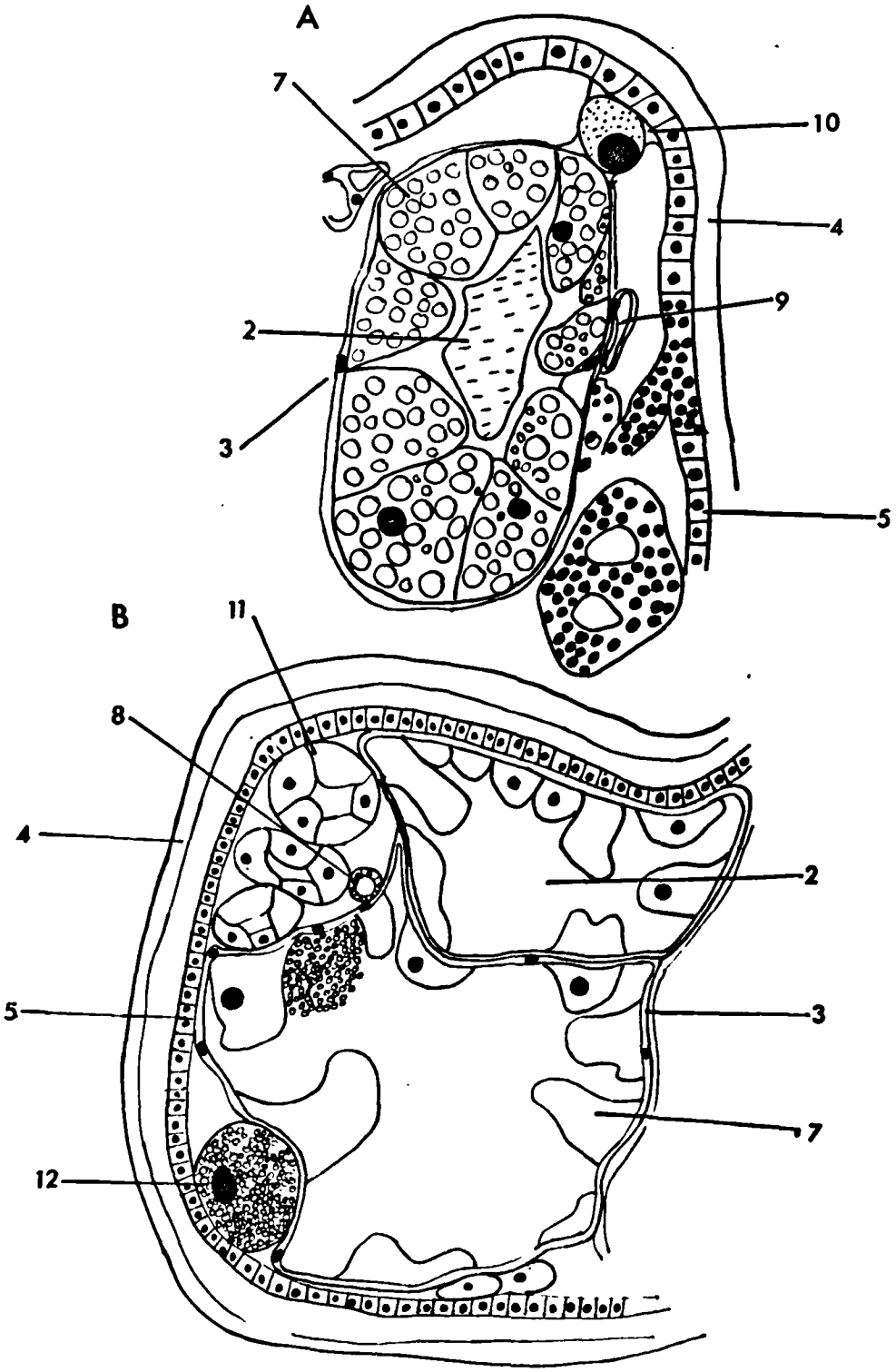

A
7
10
4
9
2
3
5
B
11
8
4
2
5
3
7
12

Fig. 100: Transverse section of larval I. triangu-liceps showing changes in the gut during feeding: showing the liberated epithelial cells in the gut lumen, (48 hours after attachment).(180×),

Same legend as Fig. 99.

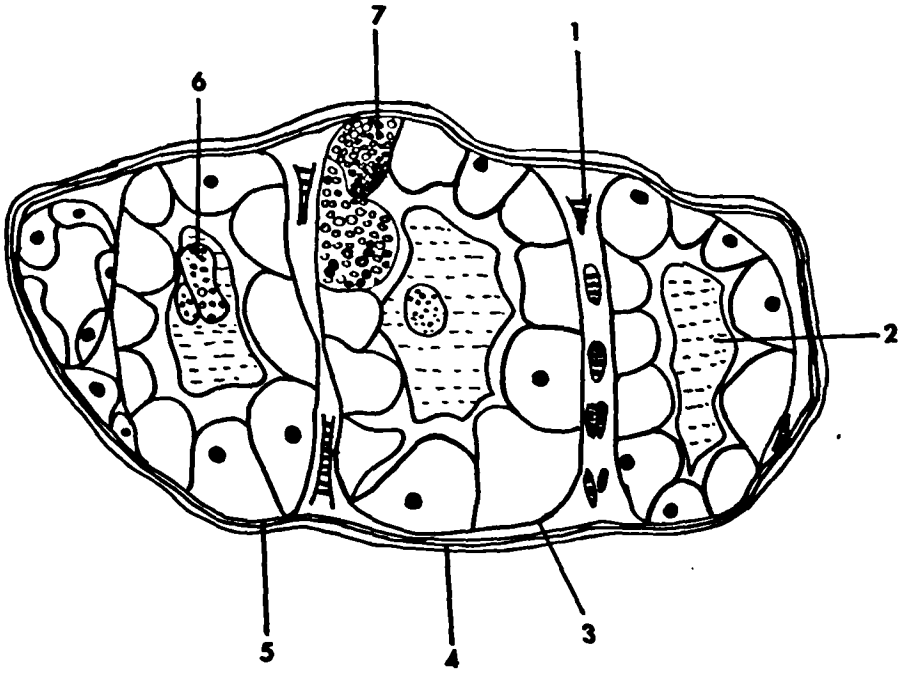

7
1
6
2
5
4
3

Fig. 101: Various metabolic products which are liberated into the gut lumen of I. trianguliceps during digestion of the blood meal.

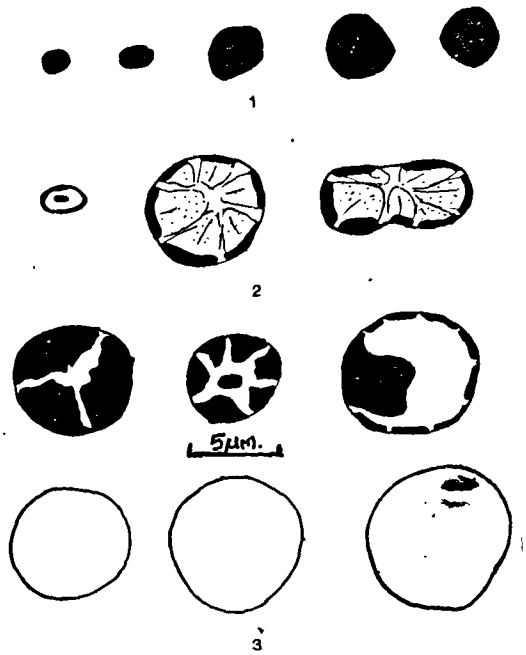


Fig. 102: Changes which occur after *B. microti* (S_2) infected blood is ingested by *I. triangulliceps*.

1. Intraerythrocytic parasites in mice.
2. Intraerythrocytic parasites in ticks.
3. Parasitaemia in mice.
4. Parasitaemia in non-haemolysed erythrocytes in the tick gut.

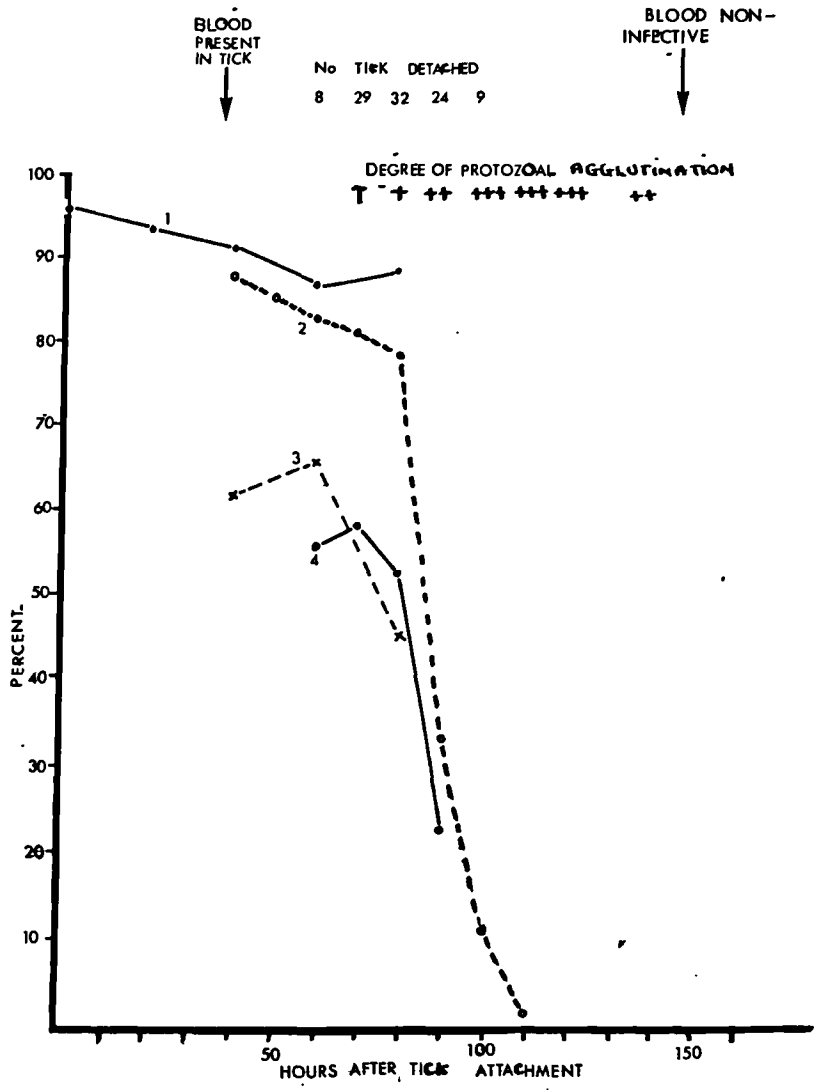

BLOOD PRESENT IN TICK
BLOOD NON-INFECTIVE
No TICK DETACHED
8 29 32 24 9
DEGREE OF PROTOZOAL AGGLUTINATION
T - + ++ +++ +++ +++ ++
100
90
80
70
60
50
40
30
20
10
PERCENT.
1
2
3
4
50
100
150
HOURS AFTER TICK ATTACHMENT

Fig. 103: Changes in morphology which occurred in B. microti after ingestion by I. triangulicops.

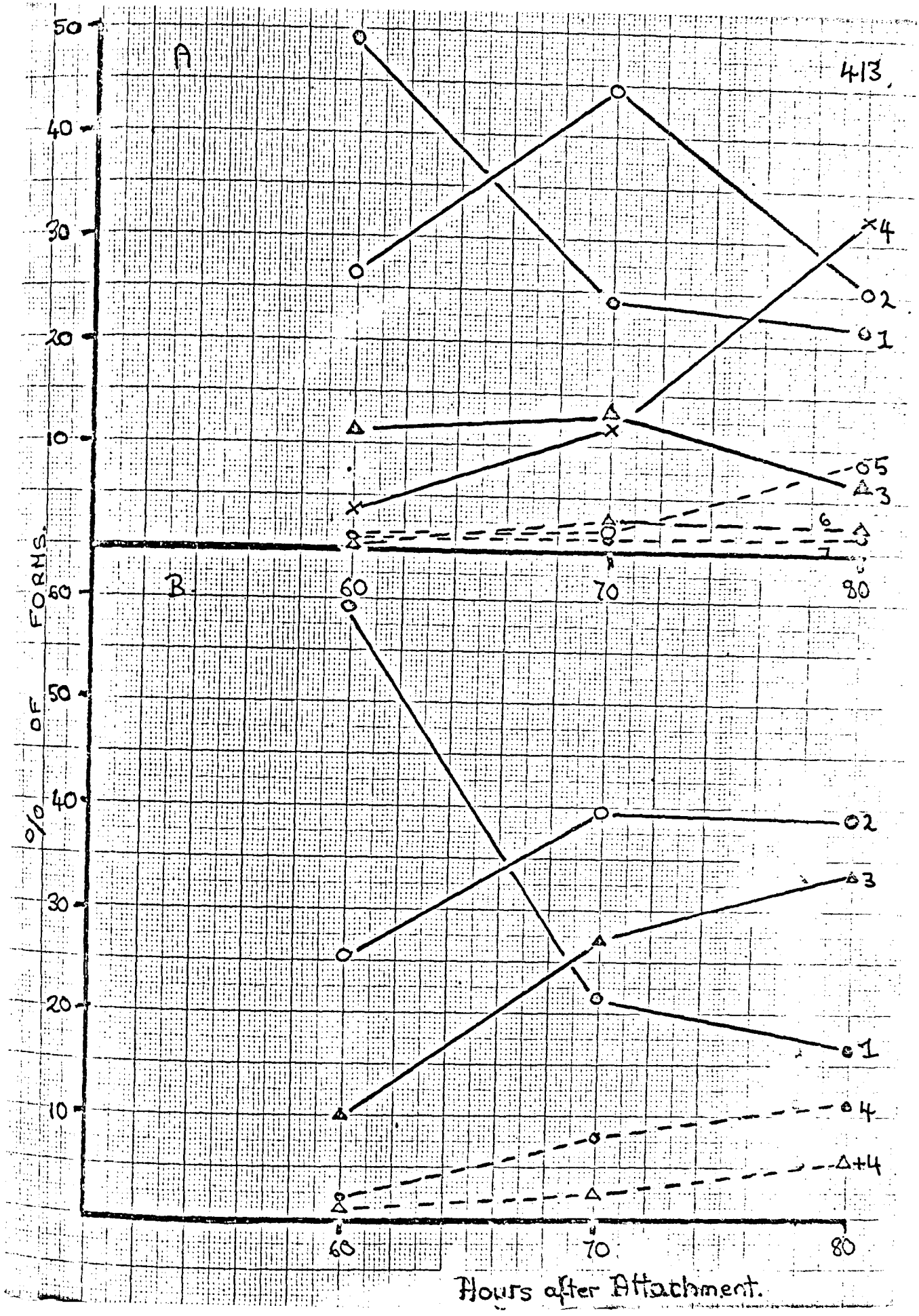
Intraerythrocytic forms

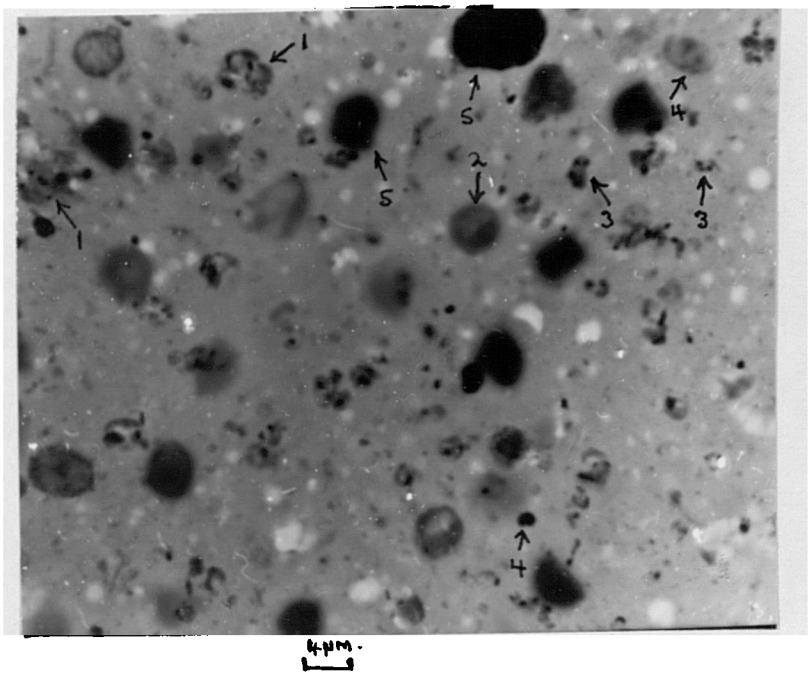
A. Morphological forms.

1 Small annular forms.
2 Large annular forms.
3 Large oval forms.
4 Small solid forms.
5 Amoeboid forms.
6 Binary fission.
7 Quaternary fission.

B. Nuclear numbers.

1 1 nucleus.
2 2 nuclei.
3 3 nuclei.
4 4 nuclei.
5 +4 nuclei.


413.
A
B
50
40
30
20
10
60
50
40
30
20
10
60
70
80
60
70
80
o/o OF FORMS.
Hours after Attachment.
1
2
3
4
5
6
+4



1 agglutinating parasites.
2 Non. haemolysed erythrocyte.
3 possible developing parasites
4. metabolic products.
5 nuclear debris.

Fig. 104: A typical field of a gut smear of I. triangu-liceps larvae fed on B. microti infected mice 10 hours after detachment

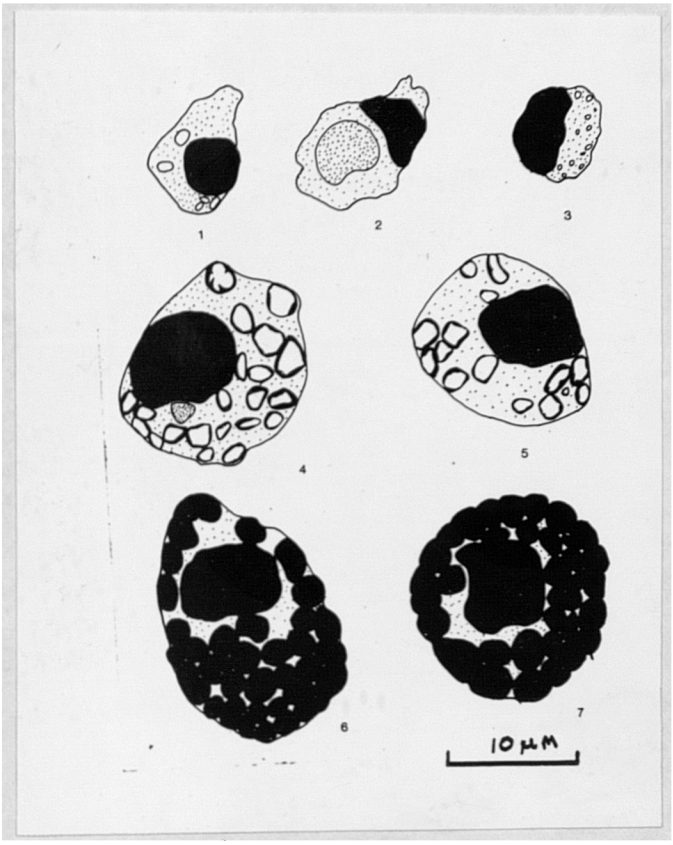


Fig. 105: Haemolymph cells of I. trianguliceps.

B. microti and B. rodhaini were studied in the guts of different species of ticks. Ticks were removed at various periods during feeding and after detachment, (Table 54). The changes of the parasites in the gut were studied by smears and sections (see Section III). The original group of red blood corpuscles were haemolysed and ruptured rapidly so that parasites remained unprotected in the gut lumen, (Fig. 102). However, the blood corpuscles in the main intake were haemolysed less rapidly, (Fig.103). The parasites within appeared to be similar in morphology to blood forms in the rodent host, (Fig. 103). At the time of attachment the parasites acquired very large vacuoles. The parasites tended to be liberated from the blood corpuscles either by haemolysis or by activity of the parasites. Twelve hours after detachment many parasites were free; lying in the lumen of the gut (Fig. 102). They tended to lose their vacuoles and had dark staining cytoplasm. At this stage, the situation in the gut became confused due to the growth of symbionts and the production of metabolic particles (Fig. 101). The gut epithelial cells appeared to liberate their contents into the lumen and epithelial cells became detached from the basal membrane. The parasites tended to become massed into groups from a couple of parasites to many thousands. Hence they appeared to agglutinate to form these clusters. By the 72nd hour after detachment of the larval ticks the parasites appeared to start to be digested. At this stage none of the parasites appeared to be infective to rodents, (Fig. 97). By the 7th day after detachment it was difficult to distinguish any parasites in the gut.

To determine the changes of the morphology of B. microti in the tick gut the parasites were analysed according to the method described in Section V.6. It was only possible to analyse the parasites at 60, 70 and 80 hours after attachment of the ticks due to the process of agglutination of the parasites. At 60 hours the parasites were very similar in morphology to the forms occurring in the rodent host, (Fig.103 c.f. Fig.38). However, after 70 hours there was an increase of large round forms which was complimentary to the decrease of the small solid forms. No significant changes in the proportion of any other forms were seen. The number of nuclei was similar to the blood forms in the mammalian host at the same time (Fig.103). However, after 70 hours a decrease in uninucleate forms was seen. This was compensated by an increase in 3 and 4 nucleate forms. After 80 hours a marked decrease of small ring and large round forms was seen which was mainly compensated by the increase of small solid forms. There also appeared to be an increase in division forms. The one nuclei parasites decreased after 80 hours which was mainly compensated by an increase of 3 nuclei parasites.

Possible stages of the cyclical development of B. microti were seen in the gut of I. trianguliceps (Fig.104). However, it is impossible to state that these were definitely part of the cyclical development in ticks. No evidence of a sexual cycle was seen in the guts of ticks. No development of B. microti was seen in the gut epithelial cells.

Haemolymph of I. trianguliceps larvae fed on B. microti infected rats was examined for parasites (see Section III). The morphology of haemolymph cells is shown in Fig.105.

There appeared to be an increase of haemolymph cells during feeding and after detachment from the host. However, no parasites could be detected either in haemolymph or haemolymph cells in 100 ticks sampled at different times after attachment and detachment.

The salivary glands of nymphal I. trianguliceps were examined for B. microti stages by sections and smears during the feeding of nymphs. 50 ticks were examined but no parasites could be detected.

TABLE 54 : CHANGES IN THE POPULATION OF B. MICROTI IN THE GUT OF I. TRIANGULICEPS LARVAE

Hours after attachment to host	No. of ticks sectioned	No. of tick gut smears	Appearance of the parasites
5	20	30	No parasites detected.
12	20	30	Occasional parasite similar to blood forms.
24	20	30	Few parasites seen. Similar to blood forms.
48	20	30	Numerous parasites similar to blood forms. Some free.
72	19	26	Numerous parasites. Some different from blood forms. Many free.
Hours after detachment from host			
12	15	20	Many parasites free tend to agglutinate.
24	15	17	Majority of parasites free all tending to agglutinate.
48	15	20	Parasites appear to begin to degenerate.
72	15	17	Parasites appear to have started to be digested.
120	15	19	Parasites non-infective to rodents.
168	15	20	Nearly all parasites digested.

8.2.3. Development of Babesia equi in ticks.

In order to obtain further information on the piroplasms of rodents, a study was made of B. equi in equines as Arthur (1966) had suggested that there were certain affinities between the piroplasms of rodents and B. equi and that rodents might act as a reservoir of the equine forms.

Babesia equi is of economic importance, since it causes the disease "biliary fever", in domestic horses and donkeys. Burchell's zebra (Equus burchelli) has been shown to harbour B. equi in both East and South Africa (Ross, 1907; Theiler, 1909). The potential transmission of the parasite from zebra to domestic equines has been demonstrated by Dennig (1966), who used fresh inocula of parasitized zebra blood to induce biliary fever in splenectomized horses and donkeys. Neitz (1956) lists nine tick species as vector of B. equi. In the Ethiopian region transmission of the parasites has been demonstrated by Theiler (1906), who effected transmission using the two host tick, Rhipicephalus evertsi, between the larval and nymphal stages, fed on infected horses and adults fed on susceptible horses.

As far as can be ascertained, no description has been published on the development of B. equi in the salivary glands of a tick vector. However, Brocklesby (1965) has described developing parasites which he assumed to be B. equi in salivary glands of adult R. pulchellus collected from a Burchell's zebra showing a patent B. equi infection. We have attempted to demonstrate the parasite in R. evertsi collected as engorged nymphs from Burchell's zebra infected with B. equi.

Fifty-six Burchell's zebra were shot during a cropping scheme in the North-Western region of Loliondo controlled area, close to the area where Dennig (1966) isolated B. equi from zebra. Thin blood smears were taken from them and stained with Giemsa's stain according to the method of Shute (1966). Forty zebra (71%) had patent infections of B. equi but the parasitaemias rarely exceeded 1% and were usually lower.

Tick collections were made from the zebra and engorged nymphs from Babesia infected animals were sent to the East African Veterinary Research Organisation, Muguga where they were allowed to moult under controlled conditions. They were maintained at 25°C and at a relative humidity of 85%, produced by a saturated sodium chloride solution. The resultant adult ticks all proved to be R. evertsi. Eight weeks after moulting about 40 ticks were placed in calico bags on the ears of rabbits according to the methods of Bailey (1960). Only five female and three male ticks attached to the rabbits' ears. They were allowed to feed for 4 days and were then removed and their salivary glands were dissected out and prepared for examination using the methods described by Martin, Barnett and Vidler (1964). The period of feeding and choice of organs were made by analogy to the known maturation of B. bigemina and Theileria parva (Riek, 1964; Purnell and Joyner, 1968).

Salivary glands were fixed and sectioned as described by Purnell and Joyner (1968) and the sections were stained by the Giemsa-colophonium method (Shortt and Cooper, 1948), using the Giemsa / Azur II stain described by Shute (1966), and mounted in green Euparal.

The morphology of blood forms of the parasites corresponds with that of B. equi as described by Nuttall and Strickland (1912) and Holbrook, Johnson and Maddon (1968) (Fig. 106). The salivary glands of three male ticks examined were found to be parasitized but no parasites were seen in the salivary glands of female ticks. The number of the acini of the male ticks' salivary glands infected with parasites was 2, 3, and 18 respectively. The majority of infected acini (21 out of 23) appeared to contain forms similar to the mature forms of T. parva described by Purnell and Joyner (1968). (Also see Fig. 110) They also resembled the mature forms of B. bigemina and B. caballi illustrated by Riek (1964) and Holbrook, Anthony and Johnson (1968) respectively (Fig. 107). The mature parasites distended the salivary glands and in some cases the acini merged with each other. The host cell nuclei were larger than those in normal cells (Fig. 107). Two acini contained intermediate forms and showed centres of reproductive activity (Fig. 108 & 109). These forms appeared to correspond to the "sporoblast" stage of T. parva as described by Cowdry and Ham (1932).

The parasites demonstrated in salivary glands of male R. evertsi must have originated from zebra since it is a two-host tick and larvae and nymphs feed on the same host. Transovarian transmission is unlikely because, except for a single report (Enigk, 1943), this method of transmission has not been observed in B. equi or in the genus Theileria. The only tick transmitted blood parasites detected in the host zebra was B. equi and it is therefore probable that development stages seen in the salivary glands of the ticks were those of this parasite.

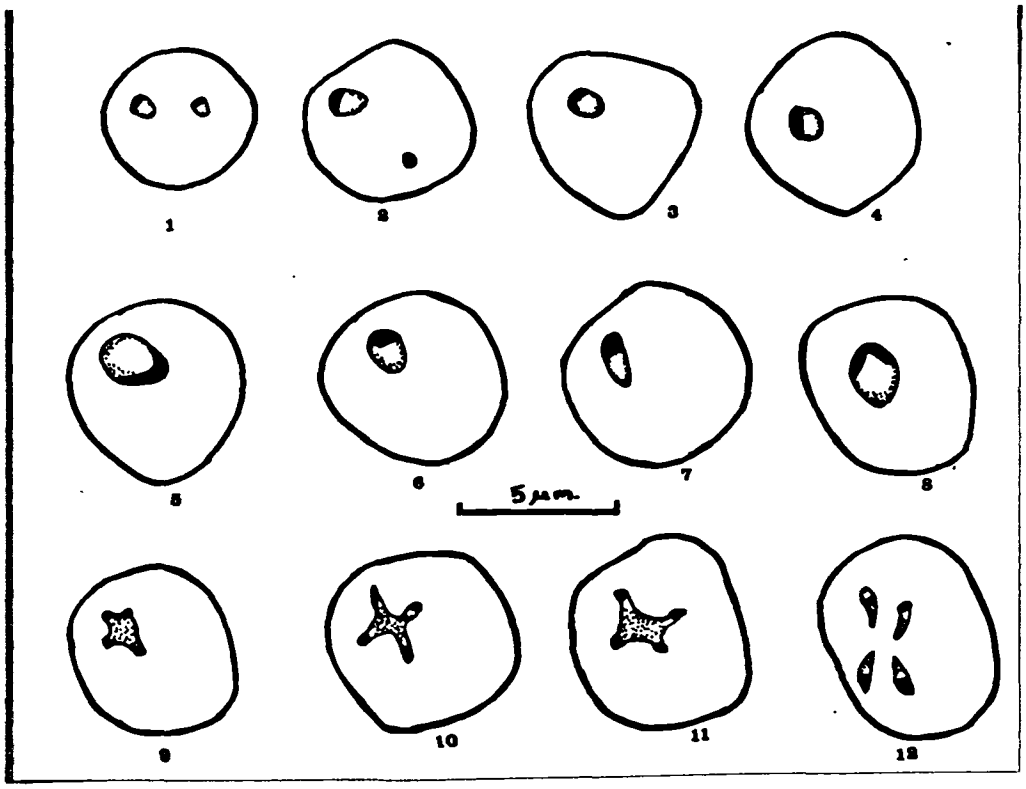
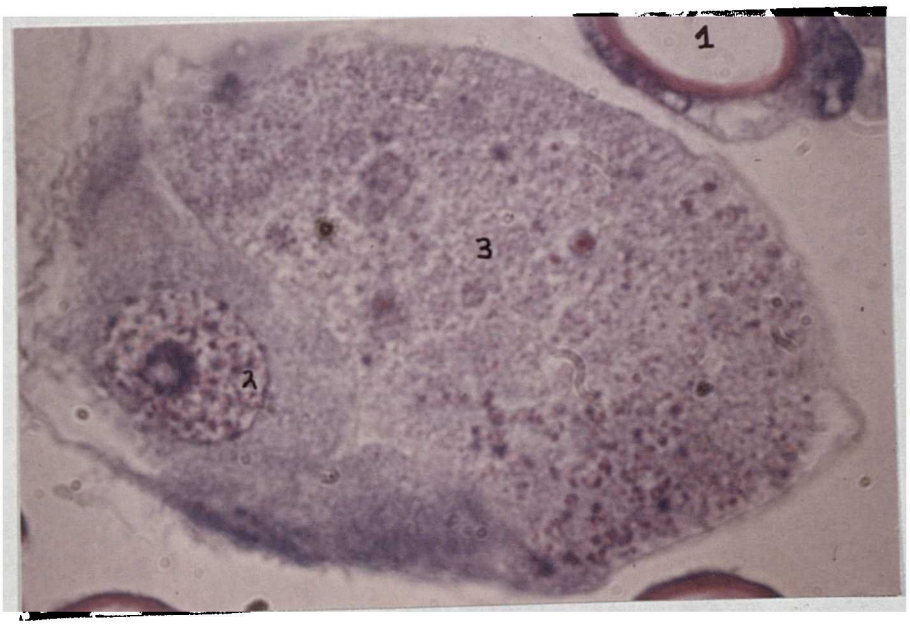


Fig. 106: The morphology of Babesia equi from zebra.



1. Salivary gland duct.
2. Large nucleus.
3. Mature stages.

Fig. 107: Babesia equi in Rhipicephalus evertsi adults.

T.S. Salivary acinus showing mature forms of B. equi. N.B. Enlarged nucleus and acinus (1600x).

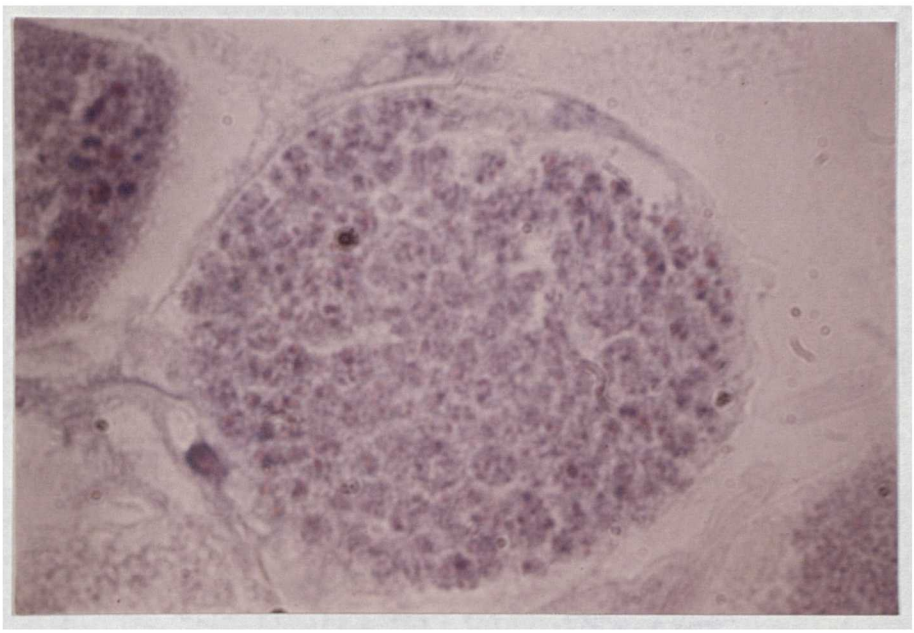
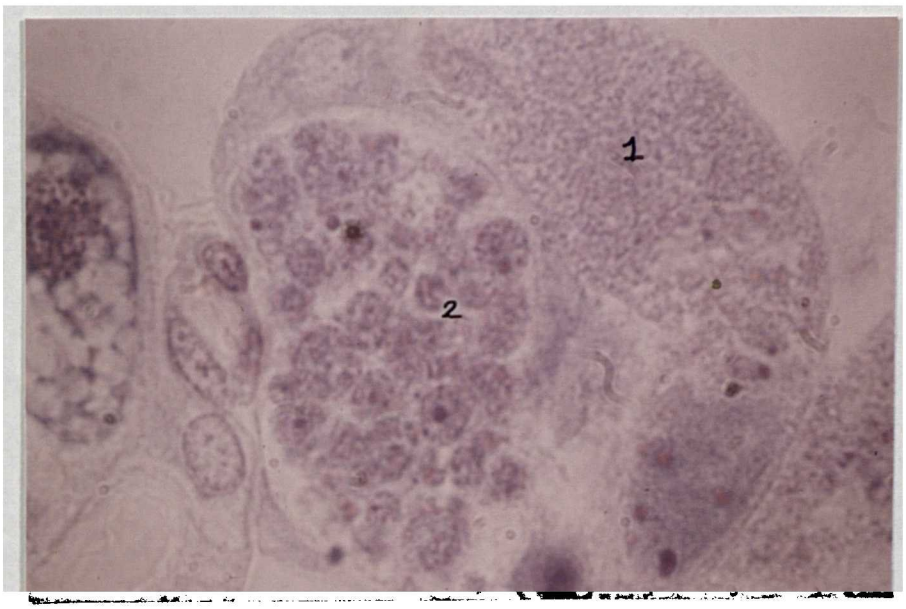


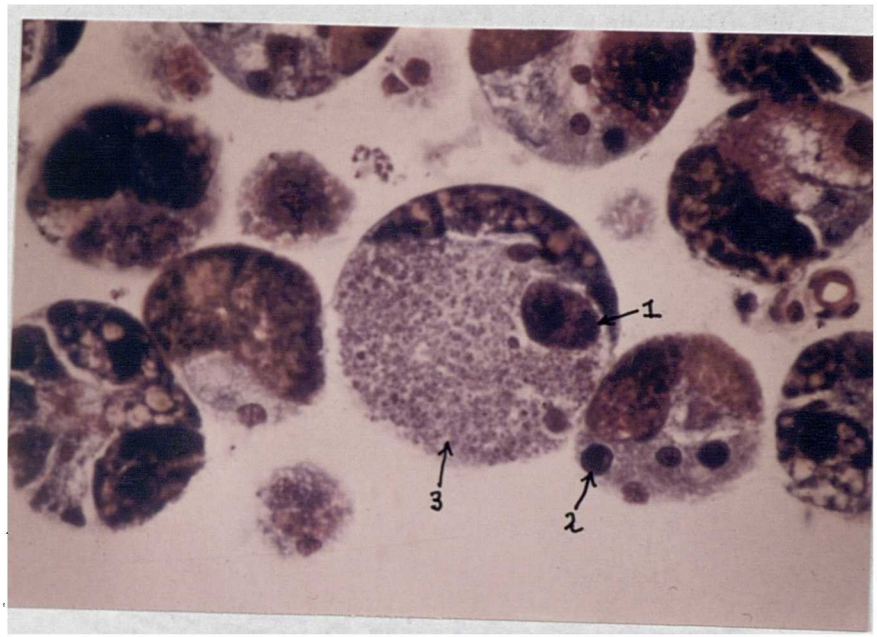
Fig. 108: Babesia equi in Rhipicephalus evertsi adults.
T.S. Salivary acinus showing intermediate stages of B. equi (x1600).



1 Mature stages
2 Immature stages.

Fig. 109: Babesia equi in Rhipicephalus evertsi adults.

T.S. Salivary acinus showing dual infection of mature and intermediate stages of B. equi (x1600).



1. Enlarged nucleus.
2. Normal nucleus.
3. Late intermediate stages.

Fig. 110: Theileria lawrencei in Rhipicephalus appendiculatus adults.

T.S. Salivary acini showing intermediate stages of Theileria lawrencei and uninfected acini (1000x). (N.B. The enlargement of the infected acinus and enlarged nuclei.)

Although B. equi parasitaemias in zebra are frequently low, the infection rate in the wild population is high (Dennig, 1966) showing that an efficient tick transmitter exists. R. evertsi were found on all 60 zebra examined in the present study and the fact that 3 out of 8 ticks that fed showed developing parasites suggests that this tick could be important in transmission of this disease.

It would appear that the majority of the parasites mature by the fourth day after attachment of the ticks since 21 out of 23 infected acini showed mature parasites by that day.

Although detailed studies of the development of Theileria in sections of the salivary glands have been made (Martin et al 1964; Purnell and Joyner, 1968), very little work on the corresponding stages of Babesia has been reported. The development of the intermediate stage, found in the present study, resembles the process of "blocking" of the cytoplasm described for T. parva by Purnell and Joyner (1968). However, according to Riek (1964) and Holbrook, Anthony and Johnson (1968), B. bigemina and B. caballi start developing in the salivary gland after the entry of the club-shaped vermicles. These forms round-off and grow during the feeding of the ticks. The chromatin material of the parasites becomes diffuse and breaks up into particles by multiple fission. These particles eventually become differentiated to form what are considered to be infective parasites. Hence no stages corresponding to the intermediate forms described by Purnell and Joyner (1968) were reported by Reik (1964) and Holbrook, Anthony and Johnson (1968). The intermediate stages of the parasite appeared to divide to produce mature forms similar to those of B. bigemina, B. caballi and T. parva.

9. Immunological studies.

Immunological studies were made on the rodent piroplasms to establish their relationship. This entailed the study of protective immunity, cross immunity, fluorescent antibody techniques and the effect of immunosuppressants and splenectomy.

9.1. Protective immunity.

The duration of protective immunity after infection and recovery is important in the epidemiology of small mammal piroplams since it will affect the proportion of susceptible hosts in a wild population (see Section V.7).

In Babesia infections, protective immunity may take one of two forms (a) sterile immunity (immunity without the presence of parasites) or (b) non-sterile immunity or premunity (immunity with the presence of parasites). It was thought until recently that the only form of immunity in Babesia infections was premunity (see Neitz, 1956; Riek, 1963). However, sterile immunity has been observed by Callow (1964) for B. bigemina, Cox and Young (1969) for B. microti and B. rodhaini in mice and Phillips (1969) for B. rodhaini in rats.

The duration and nature of protective immunity in wild and laboratory hosts was investigated.

Duration of infections.

The duration of infections of B. microti and B. rodhaini in laboratory and wild hosts is described in Section V.6. The elimination of parasites from hosts appears to vary and it is not a prerequisite for immunity (see V. 9.3.).

Duration of protection against homologous strains of B. microti and B. rodhaini.

The duration of protection with homologous strains of B. microti and B. rodhaini in mice is described in Section V.6 and V.9.3. This phenomenon was investigated in wild hosts to ascertain whether protection against homologous strains of B. microti differed from that produced in mice. It was impossible to test this phenomenon in rats because of age resistance (see Section V.6).

Ten Clethrionomys glareolus and 10 Apodemus sylvaticus were infected with 6.4×10^6 B. microti (strain S_2) parasites and allowed to recover. One animal from each group was challenged every month with approximately 10×10^6 B. microti (S_2) parasites and the resultant parasitaemias estimated. Most wild hosts appeared to be immune to challenge for at least 10 months but in some cases this immunity appeared to break down before 10 months.

Similar groups of 10 C. glareolus and 10 A. sylvaticus were infected with 6.4×10^6 B. microti (S_2) parasites. Parasites were still detectable in most hosts 10 months after infection as recrudescence occurred after splenectomy.

9.2. Cross immunity between strains of B. microti.

These experiments were designed to establish whether B. microti strains isolated from different wild hosts provided protective immunity against each other. Callow (1964) found that certain strains of B. bigemina did not cross-protect against infections

while Phillips (1969) suggested that recrudescence populations of B. rodhaini in rats differed immunologically from the initial infections. Shortt and Blackie (1965) found that considerable cross-immunity existed between strains of B. microti isolated from different species of small mammals.

The situation was complicated by the lack of suitable laboratory hosts for these experiments. The phenomenon of age resistance of rats to B. microti infection precluded the use of these hosts for cross-immunity experiments (see Section V.6). However, age resistance was not noted in mice (see Section V.6) but it was found that mice were initially refractive to infections of B. microti until a certain number of blood passages in rats had been completed. Eventually, three strains of B. microti were adapted to mice (S_1 Clethrionomys glareolus, S_2 Microtus agrestis and S_3 Apodemus sylvaticus). An experiment was designed to test the cross-immunity of these strains in mice.

Nine groups of 5 mice, which weighed between 20-25 gm. were used. Two groups were each inoculated with approximately 1×10^7 infected corpuscles of strains S_1, S_2 and S_3. Three groups of mice which were inoculated with a similar number of non-infected blood corpuscles in Alsever's solution were used as controls. The development of the parasitaemia was estimated. After 30 days the groups were challenged with 2.0×10^7 B. microti parasites and the resulting parasitaemias estimated.

The results of infections are shown in Fig. 110, 111. It is apparent from these results that strain S_1 protected against challenge with S_2, S_2 protects against

Fig. 110b Cross-immunity between strains of Babesia microti in mice.

A. B. microti (S_1) against B. microti (S_2).

B. B. microti (S_2) against B. microti (S_3).

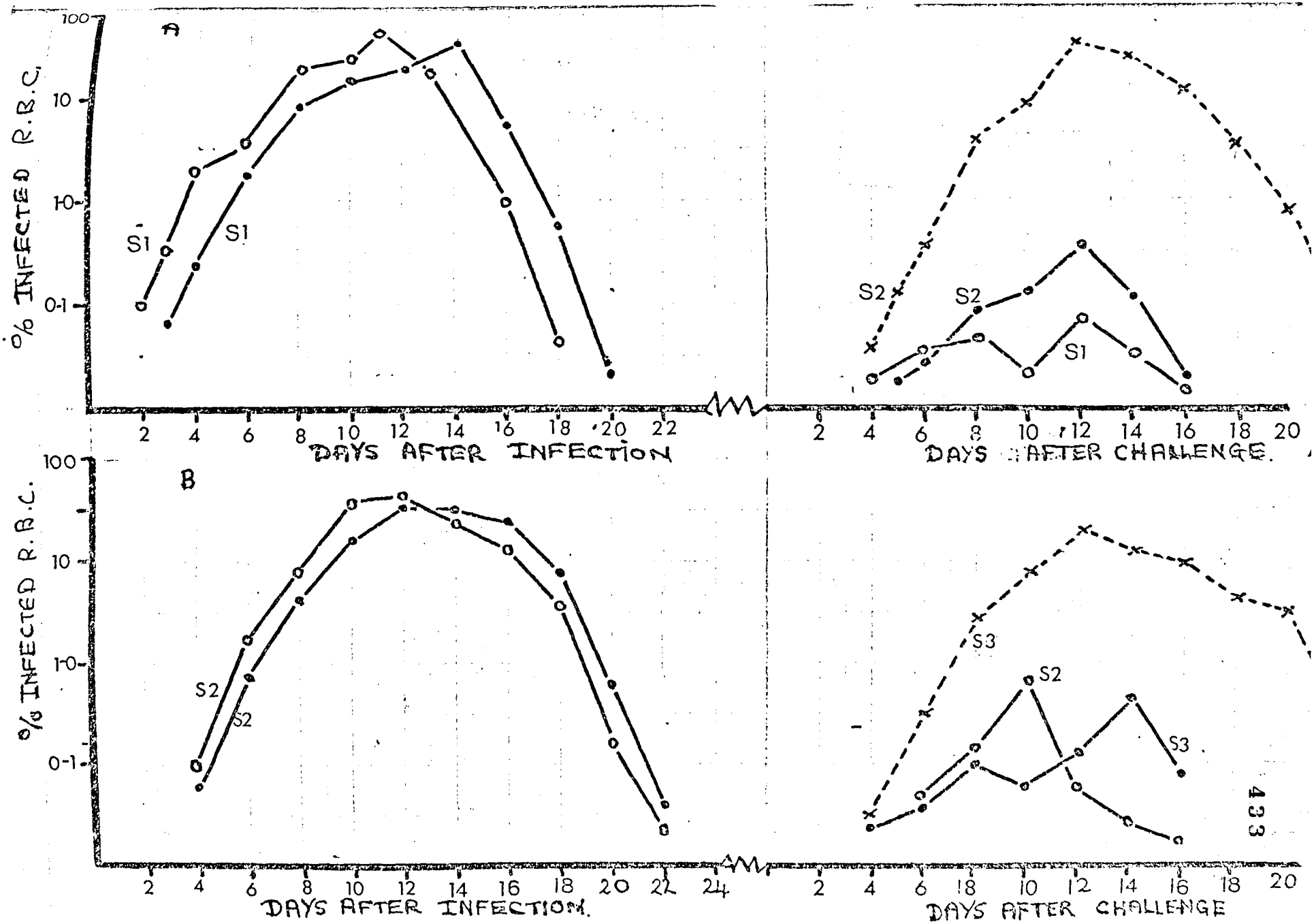
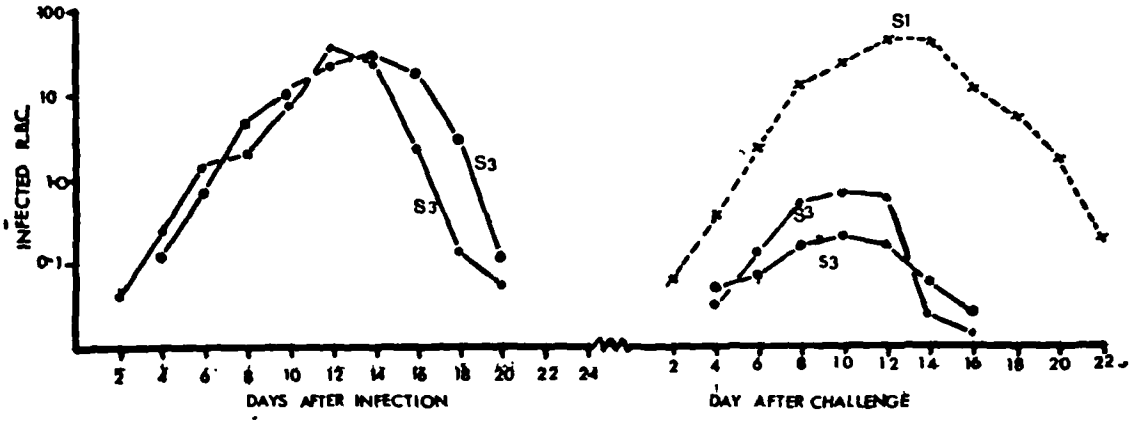

A
% INFECTED R.B.C.
100
10
1·0
0·1
S1
S1
2 4 6 8 10 12 14 16 18 20 22
DAYS AFTER INFECTION
S2
S2
S1
2 4 6 8 10 12 14 16 18 20
DAYS AFTER CHALLENGE.
B
% INFECTED R.B.C.
100
10
1·0
0·1
S2
S2
2 4 6 8 10 12 14 16 18 20 22 24
DAYS AFTER INFECTION.
S3
S2
S3
2 4 6 18 10 12 14 16 18 20
DAYS AFTER CHALLENGE

Fig. 111: Cross-immunity between strains of Babesia microti in mice.

B. microti (S_3) against B. microti (S_1).


100
10
1
0·1
INFECTED R.B.C.
S3
S3
S1
S3
S3
2 4 6 8 10 12 14 16 18 20 22 24
DAYS AFTER INFECTION
2 4 6 8 10 12 14 16 18 20 22
DAY AFTER CHALLENGE

challenge with S3 and that S3 protects against challenge with S1. The degree of protection appears to be similar between strains of B. microti from different species of hosts. Therefore it would not appear to be possible to separate these strains of B. microti on the occurrence of cross-immunity.

The results of other experiments to test cross-immunity between different strains of B. microti are shown in Table 55. It can be seen that all other strains tested in laboratory and wild hosts gave cross-immunity against each other.

Therefore it was impossible to separate strains of B. microti on cross-immunity which appeared to be complete between different strains.

9.3. Cross-immunity between Babesia microti and Babesia rodhaini in mice.

A complete series of experiments were performed to establish whether or not there was any cross-immunity between B. microti and B. rodhaini. These experiments were performed for two reasons. Firstly to see if there was any immunological relationship between the two parasites and secondly as part of a much larger programme concerned with heterologous immunity in general. The results have already been published (Cox and Young, 1969) and are appended to this thesis. The results obtained showed that both homologous and heterologous immunity between these existed but that it was strongest in the homologous situation.

Table. 55. In vivo cross-immunity between strains of B. microti isolated in Britain

		Challenge infection.						
	Hosts.	S_1	S_2	S_3	S_4	S_5	S_6	S_7
S_1 (C. glareolus.)	mice.	+++	+++	+++	—	—	—	—
	C. glareolus	+++	+++	+++	—	+++	—	+++
S_2 (M. agrestis).	mice	+++	+++	+++	—	—	—	—
	M. agrestis	+++	+++	+++	+++	—	—	+++
S_3 (A. sylvaticus)	Mice.	+++	+++	+++	—	—	—	—
	A. sylvaticus	+++	+++	+++	+++	—	—	+++
S_4 (C. glareolus)	C. glareolus	—	+++	—	+++	+++	—	—
S_5 (M. agrestis.)	M. agrestis	+++	+++	—	—	—	—	+++
S_6 (C. glareolus.)	C. glareolus	—	+++	—	—	+++	—	—
S_7 (S. minutus).	—	—	—	—	—	—	—	—

9.4. Fluorescent antibodies studies.

A direct fluorescent antibody technique was devised and used for the identification of piroplasms in the blood of mammals and in the gut of ticks. The same technique was used in an attempt to determine the antigenic similarities and differences between species and strains of Babesia and between Babesia and other parasites.

Production of antigens.

In order to produce antisera in rabbits a pure as possible B. microti antigen had to be produced. The methods used were similar to those described by Zuckeman and Spira (1962) and Diggs (1966) for the production of Plasmodium antigens.

The blood of 20-30 mice which had a B. microti infection 50% parasitaemia was obtained by cardiac puncture and pooled in Alsever's solution. The erythrocytes were sedimented by centrifugation at 1000 x g for 10 minutes at 0°C-4°C. The remainder of the procedure was carried out at the same temperature and under conditions as sterile as possible. The cells were then washed three times in 10 volumes of 0.15 M NaCl and centrifuged at 1,000 x g for 10 minutes. The buffy coat (leucocytes) was removed at each wash. The packed washed cells were then suspended in 20 volumes of cold Saponin (5 mg/ml in 0.15M NaCl). This resulted in complete haemolysis. The supernant was usually clear so that there was no evidence of any additional haemolysis. The parasites were examined microscopically to determine their purity, washed and centrifuged in cold phosphate

buffered saline at pH 7.2. The packed cells were then suspended in two volumes of phosphate saline buffer and subjected to sonic disruption by an ultrasonicator in four 30-second bursts. During this procedure the preparation was immersed in a dry ice/ethanol bath to prevent heating. In some cases sonication was not applied and the whole parasites were inoculated into rabbits.

Two rabbits were immunized with freshly prepared antigens which were emulsified in Freund's complete adjuvant (Difco) by combined foot pad, intraperitoneal and subscapular routes. The first rabbit received three inoculations at two-weekly intervals while the second rabbit received inoculations of monthly intervals. The titre produced in rabbits was estimated by immunodiffusion and microfluorescent conjugate methods. When the titre produced in rabbits reached a suitable level the rabbits were bled.

Production of fluorescent antibody conjugate.

The rabbit sera was obtained from blood in the normal way and stored at 0°C overnight. The globulin fraction was separated by precipitation with ammonium sulphate (Cambell, et al, 1964). The protein content of the globulin fraction was estimated using a spectrometer (Cambell et al, 1964).

The globulin was labelled with fluorescein isothiocyanate (B.D.H. celite dispersed) to give a fluorescein protein ratio of 1 : 1·23 after passage through a Sephadex G.25 column. The fluorescent antibody conjugate was purified to reduce non-specific staining. This was done by liver tissue powder produced by the treatment of rat liver with acetone (Cambell et al, 1964). 100 mg of powder

was used for each ml. of fluorescent antibody conjugate, mixed in a centrifuge tube and left for 30 mins. at 0°C and then centrifuged. The supernant was removed. Blood was taken from non-infected mice. The cells were washed and centrifuged three times. 0.1 ml. of packed, washed R.B.C. was added to 1 ml. of fluorescent antibody conjugate. The mixture was allowed to stand for 30 mins. and centrifuged. The fluorescent antibody conjugate was then frozen in 0.2 ml aliquotes at 20°C until use.

The production of slide antigen.

The slide antigen used consisted of blood parasites within the blood corpuscles. The parasites used were usually in rat blood corpuscles to cut down the non-specific staining since the original antigen had been produced in mice. The blood was taken from animals during a rising parasitaemia. This was done to overcome the problem of bound antibodies. The cells used were washed with buffered phosphate saline pH 7.2 to remove the sera. The cells were then resuspended in phosphate saline and smeared on grease-free slides. They were then air dried and stored at -20°C until use. The slides were fixed before use in acetone or 0.1% HCl for five minutes, and washed with phosphate buffered saline.

Staining of slide antigen.

The conjugate was applied to the slide (0.2 ml) for 30 minutes. The slides were washed in phosphate buffered saline pH 7.2 for 5 minutes and mounted in 10% Glycerine in phosphate buffered saline under a cover slip.

The fluorescent microscope used was a Zeiss with an Osram HBO-200W mercury vapour bulb and BG3 UV and Nos.

TABLE 56: SHOWING TITRES PRODUCED BY FLUORESCENT CONJUGATE B. MICROTI (S_2) RABBIT ANTISERA AGAINST VARIOUS BLOOD PARASITE ANTIGENS

Antigens and Host	Reciprocal of Fluorescent Antibody Titre
B. microti S_2 rats	128
B. microti S_1 rats	128
B. microti S_3 rats	64
B. microti S_4 rats	64
B. microti S_5 rats	128
B. microti S_6 rats	128
B. microti S_7 rats	64
B. rodhaini mice	16
B. rodhaini rats	8
B. divergens cattle	4
Plasmodium berghei mice	4
P. vinckei mice	2
Trypanosoma lewisi rats	2

40-51 barrier filters. The slides were examined with X40 objective. The end point fluorescence was evaluated by practice until consistent results were obtained.

The conjugate was titrated against homologous strains of B. microti antigens. Two fold dilutions were made using phosphate buffered saline and the titre recorded was the last dilution at which fluorescence was detected.

The highest titre was obtained with homologous ~~and~~ and heterologous strains of B. microti; the next highest with B. rodhaini antigens while only low titres were obtained with Plasmodium berghei, P. vinckei and Trypanosoma lewisi.

The results obtained are shown in Table 56.

Similar results have been obtained by Cox and Turner (personal communication) using a more specific indirect fluorescent antibody technique. Their relevant figures are these:-

Antigen	Anti-B. microti Reciprocal antibody titre
B. microti S_2	1,280
B. rodhaini	20
P. berghei	20
P. vinckei	20

Detection of parasites in the guts of ticks.

The fluorescent antibody conjugate was used to detect parasites in the gut of ticks. It was thought that this might be a useful technique to provide evidence that structures occurring in the gut were actually

parasites.

Smears were made of engorged I. trianguliceps containing B. microti. They were fixed in HCl in a similar manner to blood smears. The conjugate was diluted $^{1}/_{20}$ with phosphate buffered saline and applied to smears for 30 mins. and the slides washed. The smears were observed under the fluorescent microscope but it was found that a vast amount of background staining occurred. Therefore, no advantage was found using this method rather than using Giemsa stain!

9.5. The effect of betamethasone on rodent piroplasm infections.

One of many effects of cortisone and its synthetic derivative, prednisolone and betamethasone, is to inhibit antibody production. The synthetic compounds are considerably more active in the depression of antibody production than cortisone. The effect of corticosteriods on immunity is reviewed by Kass and Finland (1953), McMaster (1961) and McMaster and Franzl (1961).

In this study experiments were designed to ascertain the effect of corticosteriods on the course of infection of B. rodhaini and B. microti in mice and rats and to determine if these substances could be used to cause recrudescences of parasites in wild animals and therefore be used as a diagnostic tool.

Four experiments were completed to determine the effect of betamethasone on B. rodhaini and B. microti infections in mice and rats. For each experiment 4 groups of 5 animals were used. Betamethasone ("Betsolan", Glaxo Laboratories Ltd.) was inoculated intramuscularly in doses of 0.1 mg. in 0.05 ml. saline for mice and 0.4 mg in 0.1 ml saline for rats. Control groups of animals

were given a similar inocula of saline. In each experiment there was (a) a control group, (b) a group which was inoculated with betamethasone before infection, (c) after infection, and (d) after recovery. Each animal was inoculated with approximately 5 x 10^6 parasites in homologous species blood.

B. microti in rats.

The results of the various treatments are shown in Fig. 112.

The administration of betamethasone before or at the beginning of infection enhanced the parasitaemia which also remained higher than in the control animals. The fatality of infection was also increased. Administration of betamethasone after the peak parasitaemia only slightly enhanced the infection (Fig.112).

B. microti in mice.

Similar results were obtained to those in rats (Fig.113). The parasitaemia produced in the group in which betamethasone was administered before and during infections was much higher and longer in duration than in the controls. The number of fatalities was also increased. Betamethasone given after the peak parasitaemia did cause a slight recrudescence.

B. rodhaini in rats.

Similar results were obtained as for B. microti. The level of parasitaemia produced by the administration of betamethasone before and during infection was higher than in the controls (Fig.114). The number of fatalities was increased as compared with controls. (4 out of 5